Center For The Evaluation Of Risks To Human Reproduction

DRAFT

NTP-CERHR EXPERT PANEL UPDATE on the REPRODUCTIVE and DEVELOPMENTAL TOXICITY of DI(2-ETHYLHEXYL) PHTHALATE

August 2005

NTP-CERHR-

PREFACE

To be added after the Expert Panel meeting.

Reports can be obtained from the web site (http://cerhr.niehs.nih.gov**) or from:**

Michael D. Shelby, Ph.D.
NIEHS EC-32
PO Box 12233
Research Triangle Park, NC 27709
919-541-3455
shelby@niehs.nih.gov

A Report of the CERHR DEHP Update Expert Panel:

Robert Kavlock, Ph.D., Chair	US Environmental Protection Agency
Dana Barr, Ph.D.	Centers for Disease Control
Kim Boekelheide, M.D., Ph.D.	Brown University
William Breslin, Ph.D.	Eli Lilly & Company
Patrick Breysse, Ph.D.	The Johns Hopkins University
Robert Chapin, Ph.D.	Pfizer, Inc.
Kevin Gaido, Ph.D.	CIIT Centers for Health Research
Ernest Hodgson, Ph.D.	NC State University
Michele Marcus, Ph.D.	Emory University
Katherine Shea, M.D., M.P.H.	UNC School of Public Health
Paige Williams, Ph.D.	Harvard School of Public Health

With the Support of CERHR Staff:

NTP/NIEHS

Michael Shelby, Ph.D.	Director, CERHR
Christopher Portier, Ph.D.	Associate Director, National Toxicology Program

Sciences International, Inc.

Anthony Scialli, MD	Principal Scientist
Annette Iannucci, MS	Toxicologist
Gloria Jahnke, DVM	Toxicologist
Jessie Poulin	Analyst

Note to Reader:

This report is prepared according to the Guidelines for CERHR Panel Members established by NTP/NIEHS. The guidelines are available from the CERHR web site (http://cerhr.niehs.nih.gov/). The format for Expert Panel Reports includes synopses of studies reviewed, followed by an evaluation of the Strengths/Weaknesses and Utility (Adequacy) of the study for a CERHR evaluation. Statements and conclusions made under Strengths/Weaknesses and Utility evaluations are those of the Expert Panel and are prepared according to the NTP/NIEHS guidelines. In addition, the Panel often makes comments or notes limitations in the synopses of the study. Bold, square brackets are used to enclose such statements. As discussed in the guidelines, square brackets are used to enclose key items of information not provided in a publication, limitations noted in the study, conclusions that differ from authors, and conversions or analyses of data conducted by the Panel.

The findings and conclusions of this report are those of the expert panel and should not be construed to represent the views of the National Toxicology Program.

Abbreviations

ADP	adenosine diphosphate
ANCOVA	analysis of co-variance
ANOVA	analysis of variance
AUC	area under the concentration–time curve
BMD_{10}	benchmark dose, 10% effect level
$BMD_{1\ SD}$	benchmark dose, 1 control standard deviation
BMDL	benchmark dose 95th percentile lower confidence limit
BrdU	bromodeoxyuridine
bw	body weight
CAS RN	Chemical Abstracts Service Registry Number
cDNA	complementary deoxyribonucleic acid
CERHR	Center for the Evaluation of Risks to Human Reproduction
CI	confidence interval
C_{max}	maximum concentration
CYP	cytochrome P450
DEHP	di(2-ethylhexyl) phthalate
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
ECMO	extracorporeal membrane oxygenation
EPA	Environmental Protection Agency
F_0	parental generation
F_1	first filial generation
F_2	second filial generation
F_3	third filial generation
fasL	fas ligand
FDA	Food and Drug Administration
g	gram(s)
FSH	follicle stimulating hormone
GC	gas chromatography
GD	gestation day(s)
GLP	Good Laboratory Practice
hCG	human chorionic gonadotropin
HPLC	high performance liquid chromatography
H-FABP	heart-fatty acid binding protein
im	intramuscular(ly)
ip	intraperitoneal(ly)
IU	international unit
iv	intravenous(ly)
kDa	kilodalton
kg	kilogram(s)
L	liter(s)
LH	luteinizing hormone
LOAEL	low observed adverse effect level
m	meter(s)
m^3	cubic meter(s)
M	molar

MEHP	mono(2-ethylhexyl) phthalate
mM	millimolar
mmol	millimole(s)
mol	mole(s)
MRL	minimal risk level
mRNA	messenger ribonucleic acid
MS	mass spectrometry
MT	metallothionein
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
NADH	nicotine-adenine dinucleotide, reduced
ng	nanogram(s)
NHANES	National Health and Nutrition Examination Survey
NIEHS	National Institute of Environmental Health Sciences
NIH	National Institutes of Health
nmol	nanomole(s)
NOAEL	no observed adverse effect level
NTP	National Toxicology Program
OR	odds ratio
PARP	poly(ADP-ribose) polymerase
PCNA	proliferating cell nuclear antigen
PMSG	pregnant mare serum gonadotropin
PND	postnatal day(s)
PPAR	peroxisome proliferator-activated receptor
ppb	parts per billion
ppm	parts per million
PVC	polyvinyl chloride
RAR	retinoic acid receptor
RIA	radioimmunoassay
RNA	ribonucleic acid
RT-PCR	reverse transcriptase-polymerase chain reaction
RXR	retinoic acid X receptor
sc	subcutaneous(ly)
SD	standard deviation
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
$t_{1/2}$	half-life of elimination
T_{max}	time to maximum concentration
TI	tolerable intake
TNF	tumor necrosis factor
TUNEL	terminal deoxynucleotidal transferase-mediated dUTP nick-end labeling
US	United States
ZnT-1	zinc transporter-1
μg	microgram(s)
μL	microliter(s)
μm	micrometer(s)
μM	micromolar
μmol	micromole(s)
2-EH	2-ethylhexanol
2-EHA	2-ethylhexanoic acid
8-Br-cAMP	8-bromo-cyclic adenosine monophosphate

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1.0 USE AND HUMAN EXPOSURE

The first section of CERHR Expert Panel Reports is devoted to chemistry, use, and human exposure. The following conclusions regarding di(2-ethylhexyl) phthalate (DEHP) exposure were expressed by the Expert Panel in the CERHR Expert Panel Report released in 2000:

> While the Panel recognizes the variability and uncertainties in exposure estimates, it appears that for the general adult human population, ambient exposures may be on the order of 3−30 µg/kg bw/day. Non-dietary mouthing behaviors in infants and toddlers may result in exposures that are several-fold higher. The 3−30 µg/kg/d range may be increased by 2−3 orders of magnitude for infants undergoing intensive therapeutic interventions.

Since the initial CERHR Expert Panel Report on DEHP, no additional information on chemistry has been added.

Phthalates are used in a variety of products, including lubricants, perfumes, hairsprays and cosmetics, construction materials, wood finishers, adhesives, floorings, and paints. DEHP is typically added to building materials and medical devices made from polyvinyl chloride (PVC) to increase flexibility. When DEHP is used as a plasticizer in medical devices such as storage containers, bags, and tubing, it can leach from the device into infusate (e.g., pharmaceuticals, blood, blood products, parenteral nutrition solutions, air in ventilation tubing). A review by the European Commission *(1)* noted the use of DEHP in orthodontic retainers that are typically used by 7−14-year-old children. It is not known if DEHP is used in orthodontic devices in the US. The FDA referenced a study stating that DEHP has been detected as a leachate from dental composites, but that plasticizers other than DEHP are most often used for such applications *(2)*.

This section reviews the literature relating to human exposure studies published after the previous CERHR Expert Panel Report on DEHP (2000) was completed. The studies reviewed in this section included estimated or calculated exposures to DEHP and its metabolites (Figure 1) from medical devices, residential exposures, dietary exposures, and environmental exposures. Several studies reviewed the effects of temperature, contact time, and solution type in medical devices such as container bags or tubing on exposure to DEHP and various metabolites.

1.1 General population exposure

1.1.1 Exposure estimates based on DEHP levels in environmental samples
Clark et al. (Clark, 2003 #336) compiled measurements of phthalate diesters in several environmental media from databases in Canada, the US, Europe, and Japan/Asia. **[US data for DEHP are presented here.]** Many of the measurements, including those for DEHP, were compiled by Exxon Mobil Biomedical Sciences, Inc. Medians and ranges are given in Table 1 for environmental samples and in Table 2 for food samples.

In a separate paper *(3)*, the same authors presented exposure estimates using probabilistic analysis based on concentrations from an unpublished report prepared for industry. Log-normal distributions were used for most exposure sources. Estimated DEHP intakes by age group are shown in Table 3 and
Table 4. Except for intake in infants, more than 90% of estimated DEHP intake was from food. Formula-fed infants were estimated to derive 43.7% of DEHP intake from food, and breast-fed infants were estimated to derive 59.6% of DEHP intake from food. Nearly all of the remainder of DEHP intake in infants was estimated to arise from ingestion of dust.

Figure 1. DEHP Metabolism
The metabolites discussed in this report are circled. From Koch et al. *(4)*, used with kind permission of Springer Science and Business Media and of Prof. Dr. J. Angerer.

The authors indicated that exposure estimates of other authors, back-calculated based on measurements of urinary metabolites **[discussed below]**, gave lower estimates of daily intake. They suggested that the current study may have over estimated food exposure to DEHP due to use of outdated food measurements or due to failure to account for cooking-associated loss of DEHP in food. **[The Expert Panel noted that the authors summarized a number of recent estimates and all but 1 from Health Canada (1996) were within the 3–30 μg/kg bw/day range assumed in the original CERHR DEHP report. The difference in the Health Canada value is related to dust ingestion by children.]**

Table 1. Environmental DEHP Concentrations Measured in the US

Medium	Mean concentration	Median concentration (range)
Surface water, μg/L	0.21	0.05 (<0.002–137)
Ground water, μg/L	15.7	15.7 (not detected–470)
Drinking water, μg/L	0.55	0.55 (0.16–170)
Sediments, μg/kg	1.4	0.16 (0.00027–218)
Soil, μg/kg	0.03	median not available (0.03–1280)
Outdoor air, ng/m^3	5.0	2.3 (<0.4–65)
Indoor air, ng/m^3	109	55 (20–240)
Dust, g/kg	3.24	median not available (2.38–4.10)
Wastewater, μg/L	27	8.3 (0.01–4400)
Sludge, g/kg	0.301	median not available (0.000420–58.3)
Rainwater, μg/L	0.17	0.17 (0.004–0.68)

From Clark et al. *(5)*.

Table 2. Food Concentrations of DEHP

Food	Median concentration, µg/g (range)
Beverages	0.043 (0.006–1.7)
Cereal	0.05 (0.02–1.7)
Dairy (excluding milk)	0.96 (0.059–16.8)
Eggs	0.12 (<0.01–0.6)
Fats and oils	2.4 (0.7–11.9)
Fish	0.001 (0.00005–not given [90th percentile 0.02])
Fruits	0.02 (<0.02–0.11)
Grains	0.14 (<0.1–1.5)
Meat, not processed	0.05 (<0.01–0.8)
Milk	0.035 (<0.005–1.4)
Nuts and beans	0.045 (<0.08–0.8)
Poultry	0.9 (0.05–2.6)
Processed meat	0.45 (<0.1–4.32)
Vegetables	0.048 (0.0098–2.2)
Infant formula, powdered	0.12 (<0.012–0.98)
Infant formula, liquid	0.006 (<0.005–0.15)
Breast milk	0.062 (0.01–0.6)
Baby food	0.12 (0.01–0.6)
Other food	0.05 (<0.01–25)

From Clark et al. *(5)*.

Table 3. Estimated DEHP Intake by Age Group

Age group	Median DEHP intake (µg/kg bw/day)
Adult (20–70 years)	8.2
Teen (12–19 years)	10
Child (5–11 years)	18.9
Toddler (7 months–4 years)	25.8
Infant (0–6 months)	
Formula-fed	5.0
Breast-fed	7.3

From Clark et al. *(3)*.

Table 4. Age-Related Percentage of DEHP Intake from Environmental and Food Sources

Source	Adult (20–70 years)	Teen (12–19 years)	Child (5–11 years)	Toddler (7 months–4 years)	Infant (0–6 months) Formula-fed	Breast-fed
Outdoor air	0.0	0.0	0.0	0.0	0.1	0.0
Indoor air	1.0	0.9	1.0	0.9	1.5	1.1
Drinking water	0.1	0.1	0.1	0.1	0.7	0.0
Ingested soil	0.0	0.0	0.0	0.0	0.0	0.0
Ingested dust	4.3	4.2	5.0	6.6	54.1	39.3
Beverages[a]	11.2	5.2	3.3	2.2	0.0	0.0
Cereals	2.4	2.0	3.5	5.5	0.0	0.0
Dairy products[b]	13.2	11.7	12.2	12.9	0.0	0.0
Eggs	1.1	0.7	0.8	1.3	0.0	0.0
Fats and oils	16.9	19.1	16.5	11.1	0.0	0.0
Fish	1.6	0.8	0.7	0.4	0.0	0.0
Fruit products	0.9	0.8	1.1	1.4	0.0	0.0
Grains	13.4	16.6	18.1	11.1	0.0	0.0

[a]Excluding water; [b]excluding milk.
From Clark et al. *(3)*

Tsumura et al. *(6)* evaluated DEHP in prepackaged meals sold in convenience stores in Japan. In 16 meals purchased between August, 1999, and February, 2000, DEHP levels ranges from 346 to 11,800 ng/g food. Five of these meals contained enough DEHP that a 50-kg person would be estimated to receive more than the European Union tolerable daily intake value of 37 μg/kg bw/day. The authors evaluated 10 restaurant-prepared lunches, which are generally served in ceramic containers, and found DEHP levels of 12–304 ng/g food, with only 1 lunch having a DEHP level higher than 95 ng/g food. After an evaluation of preparation techniques, the authors concluded that higher DEHP content of the prepackaged meals was due to the use of PVC gloves in meal preparation. Further, spraying the gloves with an ethanol solution as a decontamination measure was believed to be associated with additional mobilization of DEHP from the gloves.

A Danish study measured DEHP in total diet samples, baby food, and infant formulas *(7)*. The total diet sample included foods consumed by 29 adults during a 24-hour period (excluding beverages and sweets). Baby food and infant formula samples were purchased in retail stores. Mean DEHP concentrations in the adult diets were 0.11–0.18 mg/kg diet. **[The lower value was calculated using 0 for samples below the limit of detection and the limit of detection for samples that were above the limit of detection but below the limit of quantization. The higher value used the limit of detection for samples that were below the limit of detection and the limit of quantization for samples that were above the limit of detection but below the limit of quantization.]** Baby food mean DEHP levels were 0.36–0.63 mg/kg food, and infant formula mean DEHP levels were 0.04–0.06 mg/kg reconstituted formula.

A review article by Latini et al. (Latini, 2004 #352} estimated from European Union reports that infants consuming formula would be exposed to 8–13 μg/kg bw/day from this source. Ingestion of DEHP in human milk was estimated to result in intakes of 8–21 μg/kg bw/day. This review also referred to an abstract *(8)* in which DEHP or mono(2-ethylhexyl) phthalate (MEHP) were measurable in 100% of milk or colostrum samples from 17 healthy mothers. Mean DEHP was 1.01 μg/mL (range 0.57–1.15 μg/mL). Mean MEHP was 0.68 μg/mL (range 0.28–1.08 μg/mL). **[Abstracts are noted for completeness but are not used in the evaluation process.]**

Yano et al. *(9)* measured DEHP in 27 powdered formula products obtained in 11 countries. The formulas had been produced in 12 countries (Japan, Taiwan, Vietnam, Turkey, the United Kingdom, Germany, Spain, Netherlands, New Zealand, Denmark, Ireland, and the US). Phthalate levels ranged from about 32 to 533 ng/g powder **[estimated from a graph]**. A single sample produced in Turkey contained the highest level of DEHP. Excluding this sample, the highest DEHP concentrations (averaged by country of production) were around 200 ng/g **[estimated from a graph]**. The authors estimated that a 3 kg child consuming 700 mL/d formula would receive a daily DEHP dose of 2.5–16.1 μg/kg bw, below the European Commission tolerable daily intake of 37 μg/kg bw.

In Japan, the estimated dietary daily intake of several plasticizers, including DEHP, resulting from the preparation, packaging, and storage of food in 3 hospitals was calculated by Tsumura et al. *(10)*. This study was an update of a similar study conducted in 1999 *(11)* that found a high level of DEHP contamination from disposable gloves used by the food preparers, resulting in the regulation of these gloves by the Japanese Ministry of Health, Labor, and Welfare. DEHP concentrations from the duplicate diet samples containing pre-determined amounts of protein, lipids, and carbohydrates varied by hospital and food type but almost all (62 of 63 samples) contained measurable amounts of DEHP. The average daily DEHP intake was 160 μg/day **[3.2 μg/kg bw/day based on a 50 kg body weight]**, which was lower than the 1999 average daily intake of 519 μg/day and lower than the tolerable daily intake range (40–140 μg/kg bw/day) set by the Japanese Ministry of Health, Labor, and Welfare.

Fromme et al. *(12)* measured concentrations of DEHP, dibutyl phthalate, butyl benzyl phthalate, diethyl phthalate, dimethyl phthalate, dimethylpropyl phthalate, di-n-octyl phthalate, dipropyl phthalate, and dicyclohexyl phthalate in indoor air and vacuum cleaner dust in 59 apartments and in indoor air in 74 kindergartens in Berlin. The median indoor air DEHP concentration was 156 ng/m^3 (95th percentile 390, maximum 615 ng/m^3) in apartments and 458 ng/m^3 (95th percentile 1510, maximum 2253 ng/m^3) in kindergartens. Median dust DEHP content in apartments was 703.4 mg/kg (95th percentile 1542, maximum 1763 mg/kg). DEHP accounted for more than 80% of the phthalate content of household dust. The authors estimated DEHP intakes for children assuming a body weight of 13 kg, inhalation of 5 m^3 air/day, and ingestion of dust at 100 mg/day to be 24 µg/kg bw/day, of which the largest contribution was an estimated food intake of 18 µg/kg bw/day (taken from the Canadian Environmental Protection Act). Estimated DEHP intake for adults was 5.06 µg/kg bw/day, assuming a 70 kg body weight, 23 m^3/day inhaled air, and ingestion of 10 mg/day dust. The food contribution to this estimate was 4.9 µg/kg bw/day. **[No source was given for the estimates of dust ingestion.]**

Another evaluation of DEHP and 5 other phthalates in household dust was conducted in Sweden as part of a case-control study of children with allergic disease and asthma *(13)*. Dust samples were obtained from children's rooms in 346 homes. In the 343 samples with DEHP levels above the limits of detection, the geometric mean DEHP dust concentration was 0.789 mg/g. In samples from the homes of 173 case children, the geometric mean DEHP dust concentration (95% CI) was 0.836 (0.724–0.964), and in samples from the homes of 176 control children, geometric mean dust concentration (95% CI) was 0.741 (0.643–0.855). There was no significant difference between the DEHP dust concentration in the homes of cases and controls ($P = 0.232$, *t*-test on log-transformed data).

1.1.2 Exposure estimates based on biomarkers

Estimates of DEHP exposure are often based on urinary concentrations of DEHP metabolites, particularly MEHP or its oxidation products. Urinary measures of metabolites provide an integrative measure across routes of exposure.

A study of the reproducibility of urinary MEHP concentrations was conducted by Hoppin et al. *(14)*. The study sample consisted of 46 African American women between the ages of 35 and 49 years. The women collected first morning urine samples on each of 2 consecutive days, timed to the onset of menses. Urine samples were frozen until analyzed. MEHP was determined using high performance liquid chromatography (HPLC)-tandem mass spectrometry (MS) with both daily urines of each woman evaluated in the same laboratory run. The median (range) urinary MEHP concentration was 7.3 (1.0–143.9) ng/mL. Adjusted for urinary creatinine, the median (range) MEHP concentration was 6.4 (0.4–77.3) µg/g creatinine. The intraclass correlation coefficient (95% CI) for urinary MEHP was 0.52 (0.32–0.68). The intraclass correlation coefficient (95% CI) for creatinine adjusted urinary MEHP was 0. 67 (0.49–0.79). Interperson variability was greater than intraperson variability. The authors indicated that the spot urine samples were a reliable biomarker of individual exposure, but because the urine collections were first-morning voids from consecutive days, the reproducibility represented in this study was a best-case example. Most women's pattern of exposure may be sufficiently stable to assign an exposure level based on a single first-morning urine biomarker measurement. However, the authors also noted that no data exist to correlate these monoester urinary markers to total exposure over time since the biological half-life of MEHP is around 12 hours.

The National Health and Nutrition Examination Survey (NHANES) 1999–2000 measured monoester metabolites of 7 phthalate esters in 2540 urine samples from adults and children older

than 6 years *(15)*. NHANES was updated in 2005 with data for the period 2001–2002 (n = 2782), noting that phthalate levels in the 2 periods were similar *(16)*. In 1999-2000, MEHP was found in more than 75% of the samples: 87% from 6–11 year olds (n=328), 84% from 12–19 year olds (n=753), and 76% from adults ≥20 years old (n=1461). **[The Expert Panel noted that no children under age 6 were tested in either time period, and it is most likely that MEHP would be detected in younger children.]** Data from the 2001–2002 samples are summarized in Table 5. NHANES noted that urinary MEHP levels were roughly comparable to those in previous reports **[discussed below]** for US residents *(17)*, pregnant women in New York *(18)*, and in men from an infertility clinic *(19)*. The 2001–2002 report indicated that levels of MEHP, 5-oxo-MEHP, and 5-hydroxy-MEHP, the last 2 of which were evaluated for the first time in this report, were similar to or up to 2-fold higher than samples obtained in German adults and children *(20-22)*. **[The Expert Panel noted that in the NHANES reports, levels below the detection limit were imputed by dividing the limit of detection by the square root of 2. The procedure was unlikely to skew conclusions for children, since most levels were above the limit of detection, but the Panel noted a possibility for error. The Expert Panel considers the NHANES data representative and thus generalizable to the entire US population.]**

Brock et al. *(23)* measured urinary phthalate monoesters in 19 children aged 12–18 months at a clinic visit and about 4 weeks later at a home visit. Phthalate-free adhesive collection bags were used to obtain the samples. Determinations were made using HPLC-tandem MS. Eight samples from 6 children had detectable levels of MEHP ranging from 6.1 to 47.3 ng/mL **[12–202 μg/g creatinine, calculated from data presented in the study]**.

Koch et al. *(20, 24)* estimated exposures to DEHP based on first-morning urine samples from 85 urban Germans aged 7–34 years (median age 33 years). Concentrations of MEHP and of the secondary metabolites 5-hydroxy- and 5-oxo-MEHP were used with metabolite excretion factors to estimate exposure. Levels of the DEHP metabolites measured in urine are summarized in Section 1.7. MEHP concentrations predicted a median DEHP daily intake level of 10.3 μg/kg bw/day. The range of estimated DEHP daily intake was from the limits of quantification to 165 μg/kg bw/day, with a 95th percentile estimate of 38.3 μg/kg bw/day. The authors believed that the primary metabolite, MEHP, was susceptible to contamination, and that the low urinary MEHP concentrations made it difficult to estimate accurately DEHP exposures. Concentrations of the secondary metabolites were 3–5 times higher than MEHP concentrations and gave a median DEHP intake estimate of 13.8 μg/kg bw/day with a 95th percentile estimate of 52.1 μg/kg bw/day. The secondary metabolites were considered by the authors to give a more accurate estimate of DEHP exposure, and any fluctuation in 1 metabolite was also seen in the other. Men had higher daily intake estimates than women (95th percentile 65.0 μg/kg bw/day for men and 27.4 μg/kg bw/day for women). No significant relationships were found between estimated DEHP daily intake and lifestyle habits obtained from a questionnaire.

David *(25)* argued in a letter-to-the-editor that Koch's daily intake estimate was too high. David's estimation of DEHP, based on a different MEHP molar excretion fraction, was approximately 5 times lower (median daily intake 1.76 μg/kg bw/day compared to the Koch et al. estimate of 10.3 μg/kg bw/day). Koch responded stating that conservative fractions were used because there were limited studies regarding molar extraction fractions *(26)*. In addition, Koch noted that if the higher molar extraction values were chosen and the secondary metabolites also considered, the metabolite dose would exceed 100% of the DEHP dose. Koch also pointed to his conclusion that the secondary metabolites were better predictors of DEHP exposure than was MEHP.

Table 5. DEHP Metabolites in Urine in the NHANES 2001–2002 Sample

| | n | | MEHP | | 5-hydroxy MEHP | | 5-oxo-MEHP | |
| | | Creatinine | | | | | | |
Group	Total	corrected	μg/L	μg/g creatinine	μg/L	μg/g creatinine	μg/L	μg/g creatinine
Total sample	2782	2772	4.27 (3.80–4.79)	3.99 (3.57–4.46)	20.0 (17.8–22.5)	18.8 (17.0–20.8)	13.5 (12.0–15.0)	12.6 (11.5–13.9)
Age group (years)								
6–11	393	392	4.41 (3.90–5.00)	5.02 (4.47–55.64)	33.6 (29.7–37.9)	38.3 (34.3–42.6)	23.3 (20.9–26,1)	26.6 (24.0–29.4)
12–19	742	742	4.57 (3.96–5.27)	3.53 (3.09–4.03)	24.9 (21.3–29.1)	19.2 (17.0–21.8)	17.5 (15.1–20.3)	13.5 (12.0–15.2)
20+	1647	1638	4.20 (3.63–4.86)	3.96 (3.48–4.50)	18.1 (15.7–20.9)	17.2 (15.2–19.4)	12.0 10.5–13.9)	11.4 (10.2–12.8)
Sex								
Male	1371	1367	4.31 (3.84–4.83)	3.49 (3.06–3.98)	22.0 (19.5–24.7)	17.9 (16.2–19.7)	14.5 (13.0–16.2)	11.8 (10.7–13.0)
Female	1411	1405	4.23 (3.67–4.86)	4.53 (4.01–5.11)	18.3 (15.7–21.4)	19.7 (17.3–22.5)	12.5 (10.8–14.6)	13.5 (11.9–15.3)
Race/ethnicity								
Mexican American	677	674	4.32 (3.75–4.98)	4.05 (3.57–4.61)	18.5 (16.2–21.1)	17.5 (15.9–19.2)	13.1 (11.6–14.9)	12.4 (11.4–13.5)
Non-Hispanic black	703	702	6.60 (5.57–7.82)	4.63 (3.95–5.42)	29.8 (26.1–34.1)	21.0 (18.8–23.3)	19.6 (17.1–22.5)	13.8 (12.3–15.4)
Non-Hispanic white	1216	1211	3.85 (3.37–4.40)	3.80 (3.33–4.33)	19.1 (16.7–21.9)	19.0 (17.1–21.1)	12.8 (11.2–14.6)	12.7 (11.4–14.1)

From *(16)*.

Koo and Lee *(27)* measured DEHP, MEHP, and other phthalates (diethyl, dibutyl, and benzyl butyl) in the urine of 150 Korean women age 20–73 years and 150 Korean children age 11–12 years **[method of subject selection not specified except as "hospital visitors"].** Geometric mean urinary DEHP was 12.5 ± 17 µg/L in women and 9.5 ± 8 µg/L in children **[error assumed to be geometric SD]**. Geometric mean urinary MEHP was 41.3 ± 50 µg/L in women and 13.3 ± 24 µg/L in children. Geometric mean DEHP adjusted for creatinine (µg/g creatinine) was 16.0 in women and 7.8 in children. Geometric mean MEHP adjusted for creatinine (µg/g creatinine) was 39.6 in women and 9.6 in children. The authors estimated median daily DEHP intake to be 21.4 µg/kg bw in women and 6.0 µg/kg bw in children with a 95th percentile estimated daily DEHP intake of 158.4 µg/kg bw in women and 37.2 µg/kg bw in children. They noted that more than 40% of women had an estimated daily intake above the tolerable intake level of 37 µg/kg bw/day set in 1998 by the EU Scientific Committee for Toxicity, Ecotoxicity, and the Environment.

Koch et al. *(28)* evaluated urine and serum levels of DEHP metabolites after a single oral dose of deuterium-labeled DEHP. A 61-year-old male volunteer weighing 75 kg (the senior author) consumed 48.10 mg **[641 µg/kg bw]** labeled DEHP. The DEHP was incorporated into butter and eaten on bread. Urine samples were collected prior to dosing and for 44 hours thereafter. Blood samples were collected prior to dosing and every 2 hours thereafter, for a total of 5 blood samples, the final one of which was 8 hours post-dosing. Blood was immediately centrifuged. Urine and serum samples were frozen until analyzed. MEHP, 5-oxo-MEHP, and 5-hydroxy-MEHP were determined by reverse phase HPLC-tandem MS. The peak urine concentration of labeled MEHP was 3.63 mg/L, 2 hours after the dose. The peak urine concentration of labeled 5-hydroxy-MEHP was 10.04 mg/L, and the peak urine concentration of 5-oxo-MEHP was 6.34 mg/L. The peak urinary concentrations of these MEHP oxidation products occurred 4 hours after the dose. Over the course of the 2-day study period, 47% of the DEHP dose was represented in urine (on a molar basis) by 1 of the 3 measured metabolites. On a molar basis, 7.34% of the administered DEHP dose appeared in the urine as MEHP, 24.7% of the administered DEHP dose appeared in the urine as 5-hydroxy-MEHP, and 14.9% of the administered DEHP dose appeared in the urine as 5-oxo-MEHP. Serum concentrations of MEHP were higher than those of its oxidation products at all time points, consistent with the more rapid urinary elimination of the polar metabolites. Estimated serum elimination half-lives for the 3 measured DEHP metabolites were all less than 2 hours.

Koch et al. *(29)* published a further characterization of DEHP urinary metabolites that might be useful in estimating DEHP exposure. The focus of the study was 2 ω-oxidation products, mono(2-ethyl-5-carboxypentyl)phthalate and mono[2-(carboxymethyl)hexyl]phthalate. This paper presented urine and blood measurements of 5 DEHP metabolites obtained from a single 61-year-old German male (the senior author) after oral ingestion of 3 different doses of deuterium ring-labeled DEHP in butter (doses were separated by 1 week). The proportional metabolite excretion relative to the DEHP dose did not vary by dose (Table 6). Over the first 2 days, 74.3% of the administered DEHP dose was excreted as metabolites, the most abundant of which, on a molar basis, was 5-hydroxy-MEHP (24.7% of the DEHP dose), followed in descending order by mono(2-ethyl-5-carboxypentyl)phthalate (21.9%), 5-oxo-MEHP (14.9%), MEHP (7.34%), and mono[2-(carboxymethyl)hexyl]phthalate (5.4%). The authors suggested that the use of secondary DEHP metabolites in urine would give a more accurate estimate of DEHP exposure and dose than MEHP in blood or urine. The study authors noted that serum MEHP is not a useful biomarker of DEHP exposure due to its short half life. However, they stated that serum levels were present at the same orders of magnitude as in animal studies, despite the fact that the human dose was 50–1000 times lower than in animal studies. The authors noted that if it is assumed that MEHP in blood is a surrogate for toxic potential, then DEHP would be 15–100 times more toxic in humans than in marmosets or rats.

Table 6. Urinary Metabolite Excretion 24 Hours after Oral Ingestion of DEHP

Metabolite	Estimated elimination $t_{1/2}$ (h)	DEHP dose (µg/kg bw)		
		4.7	28.7	650
MEHP	5	6.2	4.3	7.3
5-hydroxy-MEHP	10	23.1	22.7	24.1
5-oxo-MEHP	10	17.3	13.0	14.6
mono(2-ethyl-5-carboxypentyl)phthalate	12–15	15.5	19.4	20.7
mono[2-(carboxymethyl)hexyl]phthalate	24	3.7	5.2	3.8
Total percent of DEHP dose		65.8	64.6	70.5

Data expressed as percent of administered deuterium-labeled DEHP on a molar basis. From Koch et al. *(29)*.

Barr et al. *(30)* conducted a urinary metabolite study to evaluate whether the metabolites 5-hydroxy-MEHP and 5-oxo-MEHP were better biomarkers than MEHP of DEHP exposure. In the 50 (of 62) urine samples of adults and children that had detectable levels of all 3 metabolites, the average concentration of 5-hydroxy-MEHP was 4.3 times higher than the average concentration of MEHP; 5-oxo-MEHP concentration was approximately 3 times greater than the MEHP concentration. The median concentration of 5-hydroxy-MEHP was 36 ng/mL, the median concentration of 5-oxo-MEHP was 28 ng/mL, and the median concentration of MEHP was 4.5 ng/mL. Concentrations of 5-hydroxy- and 5-oxo-MEHP were highly correlated to one another (r^2=0.984), and both were correlated with MEHP (r^2=0.944 for 5-oxo-MEHP and 0.892 for 5-hydroxy-MEHP). 5-Hydroxy and 5-oxo-MEHP appear to be formed consistently within each individual subject (5-hydroxy/5-oxo ratio 1.4, relative standard deviation [SD] 22%), but there appeared to be variations between individuals in the oxidization of MEHP (5-hydroxy-MEHP/MEHP ratio 8.2, relative standard deviation 80%; 5-oxo-MEHP/MEHP ratio 5.9, relative standard deviation 74%). The authors concluded that 5-hydroxy- and 5-oxo-MEHP are "more sensitive indicators" than MEHP due to higher urinary concentrations and frequency of detection, although MEHP was considered a valid biomarker for health endpoints. The authors also noted that because NHANES used only MEHP as a biomarker for DEHP, exposure levels may have been higher than previously calculated.

Kato et al. *(31)* analyzed 127 paired human serum and urine samples for MEHP and the secondary metabolites 5-hydroxy- and 5-oxo-MEHP. The volunteers in this experiment were aged 6 years and older and had no known previous DEHP exposure (Silva, M personal communication June 29, 2005). The concentrations of the secondary metabolites were 10 times the concentrations of MEHP in urine; metabolite levels are summarized in Section 1.7. 5-Hydroxy- and 5-oxo-MEHP were excreted primarily as their glucuronide conjugates, and their concentrations were highly correlated with one another (r = 0.928, *P*<0.0001). Fewer than half of the serum samples had detectable levels of 5-hydroxy- and 5-oxo-MEHP, and unlike the urinary samples, sera contained higher concentrations of MEHP than of 5-hydroxy- and 5-oxo-MEHP. The authors noted that because lipases that convert DEHP to MEHP are present in the serum samples, MEHP concentrations may have been artifactually increased by any DEHP introduced during blood collection and storage. The authors' conclusions were similar to those of Barr et al. *(30)* that 5-hydroxy- and 5-oxo-MEHP appear to be more sensitive urinary biomarkers than MEHP of DEHP exposure, but that MEHP remains important in studying the health effects of DEHP exposure.

Becker et al. *(22)* measured MEHP, 5-hydroxy-MEHP, and 5-oxo-MEHP in first-morning urine samples collected from 254 German children aged 3–14 years. House dust samples were collected from ordinary vacuum cleaner bags and extracted with toluene for analysis of DEHP.

Questionnaires were used to collect information on age, gender, nutrition, time spent on the floor, floor coverings, furniture, urban versus rural residence, diet, and the presence of orthodontic braces. The non-creatinine-adjusted geometric mean urinary MEHP concentration was 7.91 µg/L (range 0.74–226 µg/L), the geometric mean urinary concentration of 5-hydroxy-MEHP was 52.1 µg/L (range 1.86–2590 µg/L), and the geometric mean urinary concentration of 5-oxo-MEHP was 39.9 µg/L (range <0.5–1420 µg/L). As in the previous studies by Koch et al. *(20, 24)*, urinary concentrations of 5-hydroxy- and 5-oxo-MEHP were higher than those of MEHP and correlated with one another (r=0.98). MEHP concentrations correlated significantly but less closely with 5-hydroxy-MEHP (r=0.72). Geometric mean concentrations of all 3 metabolites were 19–34% higher in boys than girls. When 2-year age blocks were considered, children at 13–14 years of age had the lowest geometric mean urinary concentration of 5-hydroxy- and 5-oxo-MEHP. The ratios of secondary metabolites to MEHP also decreased with increasing age, suggesting age-dependent metabolism. None of the factors identified by questionnaire were significant determinants of urinary DEHP metabolites. House dust contained a geometric mean DEHP concentration of 508 mg/kg **[ppm]** (range 22–5530 mg/kg). There was no correlation between house dust concentration of DEHP and urinary concentrations of MEHP (r = 0.06) or 5-hydroxy-MEHP (r = 0.00). The authors concluded that failure to show a correlation between house dust DEHP and urinary DEHP metabolites may have been due to consideration of the entire sample of children (ages ranging from 3–14 years). They proposed that evaluation of very young children, who are more likely to spend time on or near the floor, might show such a correlation. They indicated that their study had too few children in this age group to evaluate this possibility. **[The Expert Panel noted that a more plausible explanation for the lack of correlation between dust and urinary measures is because dust is not the only exposure medium.]**

Koch et al. *(21)* measured MEHP, 5-oxo-MEHP, and 5-hydroxy-MEHP in first-morning urine samples collected from 36 German nursery-school children aged 2.6–6.5 years. Four teachers and 15 parents also provided urine samples. Determinations were made using multidimensional liquid chromatography and tandem MS. Urinary concentrations in adults and children were compared using the Mann-Whitney *U*-test. The results are shown in Table 7. The authors interpreted these results as demonstrating that DEHP exposure was greater **[double]** among children than adults living in the same environment. The difference between children and adults was particularly evident when creatinine adjustment was used. The authors indicated that there was no relationship between urinary DEHP metabolite concentration in children and parental reports (by questionnaire) of mouthing activities. The authors speculated that the difference between children and adults might be attributable to dust inhalation or to differences in food phthalate exposures. The study authors concluded that exposure of children was twice as high as adults when body weight was considered, and that measures to reduce exposure of children need to be considered. The authors also suggested that using 5-oxo- and 5-hydroxyMEHP as biomarkers of exposure in children may be preferable to using MEHP because the oxidation products are present at higher concentrations and less likely to be affected by environmental contamination. **[The Expert Panel noted that this important study suggested double the exposure in pre-school aged children compared to adults living in the same environment. However, it was noted that highly significant differences between creatinine-adjusted children and adult concentrations were likely due to natural differences in creatinine excretion between children and adults. Because creatinine excretion is a function of lean muscle mass, smaller individuals excrete less than larger individuals and children excrete half the levels of adults. Thus, exposures based upon creatinine-corrected results would lead to overestimation of exposure in children compared to adults. However, creatinine-corrected results may be a reasonable surrogate for body weight-adjusted dose. The study demonstrated no correlation between biomarker-measured exposure and mouthing behavior (by report, no data shown). Oxidation to secondary metabolites was observed to be much higher in children compared**

to adults. Study authors noted the lack of toxicity data on secondary metabolites at the time this report was written.]

Table 7. DEHP Metabolites in the Urine of Nursery-school Children and Adults

Metabolite	Urine concentration, median (range) Unadjusted (µg/L)	 Adjusted (µg/g creatinine)	*P* (children vs adults) unadjusted/adjusted
MEHP			0.045/0.908
Children	6.6 (1.5–18.3)	8.7 (1.7–48.4)	
Adults	9.0 (2.6–43.1)	8.6 (3.8–26.6)	
5-hydroxy-MEHP			0.038/<0.0001
Children	49.6 (2.7–129)	55.8 (15.4–258)	
Adults	32.1 (10.7–103)	28.1 (10.9–63.6)	
5-oxo-MEHP			0.015/<0.0001
Children	33.8 (2.2–90.6)	38.3 (10.2–158)	
Adults	19.6 (4.9–55.1)	17.2 (4.5–40.9)	
Sum of the 3 metabolites			0.074/<0.0001
Children	90.0 (6.3–221)	98.8 (28.7–464)	
Adults	59.1 (21.1–201)	50.9 (20.5–124)	

From Koch et al. *(21)*.

Ten men participated in a study by Hauser et al. *(32)* in which 8 phthalate monoesters were measured monthly in urine for 3 consecutive days over the course of 3 months. The measured monoesters were the monoethyl, -butyl, -benzyl, -methyl, -octyl, -isononyl, and -cyclohexyl phthalate, and MEHP. Five of the phthalates were identified in more than 90% of the urine samples with substantial variation between day-to-day and month-to-month levels. More variation was observed for urine samples collected 1–3 months apart compared to 1–2 days apart. The authors concluded that the predictive value of a single urine measurement in characterizing exposure as high, medium, or low over the course of 3 months was highest for monoethyl phthalate and lowest for MEHP. Of men in the highest tertile for MEHP exposure based on the 3 months of urine monitoring, 56% would have been identified by a single urine sample. A single MEHP measurement would have identified 83% of men in the lower 2 tertiles. The study authors noted that the poor predictive value of MEHP levels in single urine samples could lead to misclassification of exposure over a 3-month period and bias towards the null hypothesis when assessing exposure-response relationships.

Adibi et al. *(18)* measured phthalate diesters in 48-hour air monitoring samples collected by 30 pregnant women in New York and 30 pregnant women in Krakow, Poland. The New York women were Dominican or African-American, were of low socioeconomic status, and were in the third trimester of pregnancy. The Polish women had been residents of the city for at least a year, were middle-class, and were in the second trimester. Spot urine samples were collected at the end of the personal air sampling period from 25 of the New York women and analyzed for monoester metabolites of the phthalate diesters. Spearman rank correlation was used to evaluate the relationship between the diester concentration in air and the corresponding monoester concentration in urine. All 60 personal air samples contained measurable concentrations of diethyl, di-*n*-butyl, di-isobutyl, and butyl benzyl phthalate, and DEHP. The median DEHP air concentrations (ranges) were: New York 0.22 (0.05–0.41) µg/m^3, Krakow 0.37 (0.08–1.1) µg/m^3. Median (range) urine MEHP in the subset of 25 New York women was 4.60 (1.80–449) µg/g creatinine. The study authors noted that urinary MEHP levels reported for the New York group were similar to values reported for the NHANES sample. There was no significant relationship

between urinary MEHP and air sample DEHP concentrations (Spearman correlation coefficient 0.37). **[The Expert Panel noted that the lack of association between air levels and MEHP urine levels may be due to the fact that air is not the only exposure medium.]** Significant correlations were reported for personal air samples and urinary concentrations of butyl benzyl, dibutyl, and diethyl phthalate.

Latini et al. *(33)* demonstrated placental transfer of DEHP/MEHP. They found either DEHP or MEHP in 87.5% of 24 maternal plasma samples and 76% of 25 umbilical cord samples (1 set of twins). Samples were collected at delivery at 35–42 weeks gestation. DEHP was measurable in 17 (71%) of the 24 maternal samples and 11/25 cord samples (P = 0.024, chi-squared). MEHP was measurable in 18/24 (75%) of maternal samples and 18/25 (72%) cord samples (P NS). Mean ± SD DEHP concentrations were higher in cord plasma than in maternal plasma (2.05 ± 1.47 µg/mL [n=11] compared to 1.15 ± 0.81 µg/mL [n=11], P = 0.042, t test). MEHP concentrations in maternal and umbilical cord plasma were similar with mean ± SD values of 0.68 ± 0.85 for maternal plasma and 0.68 ± 1.03 µg/mL for cord plasma. The authors concluded that variation in plasma concentrations could have been due to different environmental exposures, and that fetal bioaccumulation may have been due to lack of maturation of excretory organs. Although no statistical correlations were found between DEHP and MEHP levels in either the mother or infant, the authors noted that exposure to phthalates begins prenatally and suggested that fetal exposure is "closely related to maternal exposure."

1.2 Exposure assessed through toys

Bouma et al. *(34)* measured DEHP released from 47 PVC-containing toys bought in the Netherlands after mixing with a saliva stimulant **[composition not specified]**. DEHP was found in 20 (43%) of the 47 toys at 3–44% by weight. Migration of DEHP into saliva simulant increased with increasing DEHP content. Six toys exceeded the Dutch guidance release value (2.3 µg/min/10 cm^2) for children younger than 1 year. Six toys also exceeded the Scientific Committee on Toxicity, Ecotoxicity and the Environment migration guidance of 1.7 µg/min/10 cm^2; 5 of these toys were intended for children older than 3 years of age.

Niino et al. *(35)* identified migration into simulated saliva of DEHP from a sample of a PVC ball that contained DEHP 190 mg/g. The simulated saliva contained sodium chloride 4.5 g/L, potassium chloride 0.3 g/L, sodium sulfate 0.3 g/L, ammonium chloride 0.4 g/L, urea 0.2 g/L, and lactic acid 3.0 g/L dissolved in distilled water and adjusted to pH 6.5–7.0 with 5 M sodium hydroxide. The ball portion was shaken with the simulated saliva at 35ºC for 15 minutes, yielding DEHP 315±25.0 µg/10 cm^2/hour (mean ± SD, n = 5). When 4 volunteers **[age not specified]** chewed a ball segment for 4 15-minute sessions separated by rest periods, the amount of mobilized DEHP measured in saliva was 44.4±12.3 µg/10 cm^2/hour. Hydrolysis to MEHP occurred to a limited extent. Over the 60-minute chewing period, salivary DEHP decreased from about 42 nmol to 32 nmol while MEHP increased from 0 nmol to about 2 nmol **[estimated from a graph]**.

1.3 Exposure through Building Materials

Otake et al. *(36)* measured indoor air concentrations of common phthalates and phosphate esters in Japanese homes. Twenty-seven homes in the Tokyo metropolitan area had indoor air concentrations of DEHP ranging from <0.001 to 3.13 µg/m^3. The mean ± SD DEHP concentration was 0.32 ± 0.6 µg/m^3, the second highest concentration next to dibutyl phthalate, which was 0.75 ± 1.17 µg/m^3. DEHP levels were 100–1000 times higher than ambient outdoor air concentrations reported in articles cited by the author: 2.0 ng/m^3 in Sweden and 16 ng/m^3 in Japan.

Danish authors *(37, 38*) studied the DEHP emission and sorption characteristics of PVC flooring material in an emission cell and in an emission test chamber. Airborne DEHP concentrations increased up to 150 days, at which point emissions leveled off at approximately 1 µg/m^3. Dust on soiled PVC material increased the emission rate of DEHP; dust sorbed 3700 µg of DEHP compared to 900 µg emitted from unsoiled PVC material over 68 days. The authors concluded that resuspended dust may be an important route of DEHP exposure. A similar evaluation of PVC-coated wall coverings showed variable chamber air DEHP concentrations with a maximum air concentration of just under 1 µg/m^3 *(39)*.

1.4 Exposure through Wastewater

Marttinen et al. *(40, 41*) found DEHP to be the most frequently encountered phthalate in sewage in Finland, with DEHP concentrations of 98–122 µg/L compared to <5 µg/L for other phthalates. The highest concentration, 160–166 µg/g, was found in treated sewer sludge; this concentration exceeded the maximum value set by the European Commission for the use of sludge in agriculture. In 4 sewage treatment plants, 80–96% of DEHP was removed, but the remaining DEHP accumulated in treated sludge. In leachate from 11 landfills, DEHP was the most commonly identified of 14 measured waste chemicals. When landfill leachate was handled in the same treatment plants as sewage, the contribution of leachate DEHP to total sewage leachate was low, on the order of 1%.

1.5 Medical exposures

Loff et al. *(42)* quantified DEHP leaching from PVC infusion set tubing during infusion of parenteral nutrition, blood products, and selected pharmaceuticals at room temperature (27ºC) using neonatal intensive care (NICU) protocols employed in treating sick neonates. The highest DEHP concentration was found in lipid-containing solutions used for parenteral nutrition (424.4 µg/mL over 24 hours) resulting in an exposure of 5 mg/kg bw for a 2 kg infant (25 mL solution). Small amounts of DEHP were found in an amino acid/glucose solution (0.83 µg/mL, 24 hours). Blood products stored in 20 mL PVC bags contained 7–339 µg/mL DEHP. When the blood product in the PVC bags was administered through PVC tubing, a single 20-mL dose of a blood product for a 2-kg baby was estimated to contain 608 µg DEHP for packed red blood cells, 928 µg DEHP for platelet-rich plasma, and 552 to 8108 µg DEHP for fresh frozen plasma. **[MEHP levels were not measured.]** Administration of 1% propofol (10 mL) resulted in a daily DEHP dose of 6561 µg. Administration of 28.8 mL fentanyl resulted in a DEHP dose of 132.5 µg and administration of 24 mL midazolam resulted in a DEHP dose of 26.4 µg. The study authors concluded that the dose of DEHP for a typical preterm neonate requiring total parenteral nutrition (TPN) and additional therapy can range from 10 to 20 mg/day.

Data from the Loff et al. *(42)* study was used by the the US Food and Drug Administration (FDA) *(2)* to estimate infant exposures to DEHP through administration of sedatives (discussed below). Because propofol is not approved for sedation in pediatric patients, the intake value from fentanyl (0.03 mg/kg bw/day) was used as the upper-bound estimate of DEHP exposure of 4 kg neonates receiving conscious sedation. **[The Expert Panel notes that the FDA report was dated 2001 on the FDA web site, although the cited publication in Government Reports, Announcements, & Index was dated 2004.]**

Loff et al. *(43)* updated their previous study *(42)* by evaluating the effects of temperature on DEHP release. Temperature and contact time greatly affected the release of DEHP from PVC-infusion lines into a lipid-containing infusion solution. An increase in temperature from 27ºC (the temperature used in the earlier study) to 33ºC increased the amount of released DEHP by

approximately 30% (422 μg/mL at 27ºC and 540 μg/mL at 33ºC). The administration of 24 mL of this infusion to a 2-kg newborn resulted in a DEHP dose of 13 mg (6.5 mg/kg bw) at 33ºC compared to 10 mg (5 mg/kg bw) at 27ºC. The rate of extraction of DEHP from PVC tubing was directly related to the length of contact time between the solution and the tubing. The concentration of DEHP in the infusion solution increased from 25 μg/mL at 4 hours to 478 μg/mL between 20 and 24 hours. The authors noted that these findings were important because neonatal ICUs are typically maintained at 30º, and incubator temperatures can reach 37ºC. Loff et al. *(43)* also noted that these exposure estimates were only from 1 type of medical device, and that newborns in these units can be exposed through other devices as well.

[The Expert Panel noted limitations in the Loff et al. studies *(42, 43)*. First, the authors did not address prevention of DEHP contamination or mention if contamination was a problem. Second, because blood products contain enzymes that can metabolize DEHP to MEHP, measurement of DEHP alone may underestimate total blood product exposure to DEHP-related chemicals. Several important observations were made by the Expert Panel. The first is that even though glass containers were used to store lipophilic substances that were slowly infused through PVC tubing, significant DEHP exposure was estimated. Second, data demonstrated that at NICU temperatures (33ºC), perfusion of TPN through PVC tubing increased DEHP extraction by 20–30%, compared to extraction observed at room temperature (27ºC). Third, extraction rates increased for first 12 hours and then slowed. Lastly, rinsing lines did not affect leaching.]

Another study *(44)* measured the extent of DEHP leaching into a lipid-containing TPN solution from ethyl vinyl acetate bags with PVC connectors and tubing. The bags and tubing were stored at 4ºC for 24 hours or 1 week prior to simulated use. The 1-week storage period simulated conditions that can occur in home parenteral nutrition programs. For both storage periods, DEHP concentrations were highest in solutions with the highest lipid content (3.85%) and decreased for simulated infusions as flow rate decreased. After storage for 24 hours, DEHP content per infusion set ranged from 0.2 to 0.7 mg in the ethyl vinyl acetate bags and from 0.8 to 2 mg in the outlet tubing. The authors concluded that the DEHP dose from a TPN infusion could range from 0.8 to 2 mg/day for an infant or child depending on the lipid content and flow rate. **[The Expert Panel noted that the study provides new information on DEHP leaching during home TPN use.]**

Kambia et al. *(45)* used an HPLC method to measure the amount of DEHP leaching into lipid-containing TPN solutions stored in ethyl vinyl acetate bags with PVC outlets and infused through PVC tubing. The amount of DEHP leaching into TPN solutions was estimated at 0.2±0.008 to 0.7±0.02 mg from bags and 0.8±0.09 to 2±0.07 mg from tubing. **[Variances assumed to be SD as reported in other parts of the paper.]** DEHP was measured at 0.3–6.9 μg/mL in blood samples from 4 children receiving TPN. **[Levels of DEHP metabolites in blood were not measured.]**

PVC tubing designed to reduce DEHP leaching by using an "inert" polyethylene inner lining did not show significant differences in the amount of DEHP released into solution compared to standard PVC tubing *(46)*. Three types of multi-layer tubing (PVC, PVC/polyethylene, and PVC/ethyl vinyl acetate/polyethylene) were tested using an etoposide solution containing a polysorbate excipient. DEHP concentration increased nonlinearly with polysorbate concentration and linearly with temperature and contact time. DEHP leaching was particularly evident during the first 2 hours of contact. The authors concluded that polysorbate was responsible for the release of DEHP into etoposide solutions, and that the polyethylene linings did not prevent the release of DEHP into solutions. They noted that DEHP was found on the inert lining even before coming in contact with either solution and suggested that DEHP might gain access to the tubing

lumen through pores in lining materials. The authors suggested the use of polyethylene-only tubing for infants in incubators (37ºC) who receive solutions with polysorbate. **[The Expert Panel noted that the study demonstrates that multilayer tubing does not prevent leaching as marketed and is not a solution to DEHP exposure problems. However, failure to address possible contamination could have potentially been the reason why no differences were observed between lined and unlined tubing.]**

Haighton et al. *(47)* published an abstract in which DEHP exposure from a closed inhalation spray container was estimated at 0.0037 µg/kg bw/day. Details of the assumptions made in this estimation were not available in the abstract. **[The Expert Panel notes this abstract for completeness, but the abstract will not be considered in the evaluation.]**

Calafat et al. *(48)* conducted a study to measure DEHP exposures in infants receiving multiple treatments in the NICU. Six premature newborns undergoing intensive care interventions for more than 2 weeks were tested for the 3 DEHP urinary biomarker metabolites MEHP, 5-oxo-MEHP, and 5-hydroxy-MEHP. All 3 metabolites were found in 33 of the 41 urine samples collected from these infants. 5-Hydroxy- and 5-oxo-MEHP were found in all 41 samples, and measurements of these 2 metabolites were an order of magnitude higher than those for MEHP. Urinary concentrations varied widely among the infants. **[The Expert Panel noted that the high variability in the ratio of MEHP to oxidative metabolites suggests metabolic variation. Less than 25% of the metabolites were present in "free form," which is the putative biologically active species.]** Geometric mean 5-oxo-MEHP was 1617 ng/mL, 5-hydroxy-MEHP was 2003 ng/mL, and MEHP was 100 ng/mL. Urinary concentrations of 5-hydroxy-MEHP and 5-oxo-MEHP were highly correlated. The author notes that the geometric means found in this study were several-fold higher than the MEHP geometric mean in the general US population 6 years and older (3.43 ng/mL). **[The Expert Panel notes the high importance of this study because it is the first to quantify real-world exposures resulting from the use of multiple DEHP-containing devices in a contemporary NICU. The study documents DEHP exposures that are orders of magnitude higher than the general population, including children ≥6 years. Three metabolites were examined and metabolism in premature infants was partially elucidated. Results suggested that MEHP may not be the best marker of exposure. The study also enumerated persistent data gaps regarding possibly increased susceptibility of children, including the effects of high gastric lipase activity, lower capacity for glucuronidation, increased permeability of the blood-testis barrier, and possibly increased absorption in the gastrointestinal tract. Limitations of the study included small sample size, no measurement of exposure from individual sources, and no discussion of primary MEHP exposure (i.e., MEHP infused directly into the patient).]**

Green et al. *(49)* measured urinary DEHP metabolites in 54 infants in a NICU. The infants were hospitalized in 1 of 2 hospitals. One of the investigators observed the care of each infant for a total of 3–12 hours (1–4 hours/day on 1–3 days) and noted the products used in the care of the infants. DEHP exposure was rated low, medium, or high based on the kind of medical devices used and the length of time used. Medical records were not consulted in evaluating infant exposures. Urine was collected from diaper liners or from cotton gauze placed in the diaper. The urine was collected during the observation period. Some infants had 2 or 3 urine specimens collected; in these instances, the urinary MEHP concentrations were highly correlated within infants. Urine was assayed for MEHP, 5-oxo-MEHP, and 5-hydroxy-MEHP using HPLC-tandem MS. **[Only MEHP results were given in the paper.]** Specimens with MEHP levels below the limit of detection were assigned a value of half the limit of detection. Statistical analysis of urinary MEHP by sex, institution, and DEHP exposure group was performed using the Mann-Whitney-Kruskal-Wallis test, multiple linear regression, and quartile regression.

Urinary MEHP levels are shown in Table 8. DEHP exposure group was described as a substantial predictor of urinary MEHP levels ($P = 0.09$ after adjusting for infant sex and institution). Infants in the medium exposure group had urinary MEHP concentrations twice as high (calculated from the regression model) as infants in the low exposure group ($P = 0.3$), and infants in the high exposure group had urinary MEHP concentrations 5.1 times as high as infants in the low exposure group ($P = 0.03$). **[The Expert Panel noted that urinary MEHP levels were quite different between infants at the two hospitals and suggested that it may be due to different products used at the two hospitals.]**

Table 8. Urinary MEHP in Infants in Two NICUs by DEHP Exposure Group

	Urinary MEHP, ng/mL, by percentile		
Exposure group (n)	25th	Median	75th
Low (13)	<0.87	4	18
Medium (24)	3	28	61
High (17)	21	86	171

Exposure status assigned by observing use of medical device during 3–12 hours of the child's care.
$P = 0.001$ for exposure class (i.e., low, medium, high)
From (49).

The Expert Panel is aware of recent reviews in which exposure to DEHP through medical devices was estimated. The most thorough estimates were conducted by the FDA (2) and are summarized in Table 9. **[The Expert Panel notes that the estimates were conducted using data that were available to the CERHR Expert Panel during their first phthalates review and does not provide new data.]**

The FDA noted a lack of data to estimate exposure through breast milk for infants of mothers who had undergone or were undergoing medical procedures like hemodialysis. The FDA believed that few infants were exposed to breast milk from women undergoing these kinds of medical procedures.

Table 9. FDA Estimates of DEHP Exposures Resulting from Medical Treatments

	Estimated DEHP dose (mg/kg bw/day)	
Medical procedure	70 kg adult	4 kg neonate
Crystalloid intravenous (iv) solution infusion	0.005	0.03
Infusion of pharmaceuticals with solubilization vehicles		
Administered according to manufacturer instructions	0.04	0.03
Mixed and stored at room temperature for 24 hours	0.15	
TPN administration		
Without added lipid	0.03	0.03
With added lipid	0.13	2.5
Administered via ethyl vinyl acetate bag and PVC tubing	0.06	
Blood transfusion		
Trauma patient	8.5	
Transfusion/extracorporeal membrane oxygenation (ECMO) in adult patients	3.0	

	Estimated DEHP dose (mg/kg bw/day)	
Medical procedure	70 kg adult	4 kg neonate
Exchange transfusion in neonates		22.6
Replacement transfusions in neonates in NICU		0.3
Replacement transfusions to treat anemia in chemotheraphy and sickle cells disease patients.	0.09	
Replacement transfusions in patients undergoing coronary artery bypass grafting	0.28	
Treatment of cryodisorders with cryoprecipitate	0.03	
Cardiopulmonary bypass		
Coronary artery bypass grafting	1	
Orthotopic heart transplant	0.3	
Artificial heart transplant	2.4	
ECMO		14
Apheresis	0.03	
Hemodialysis	0.36	
Peritoneal dialysis	<0.01	
Enteral nutrition	0.14	0.14
Aggregate exposures of NICU infants undergoing iv administration of sedatives, iv administration of TPN, and replacment transfusion		2.83

From FDA *(2)*.

The studies listed below add to the knowledge obtained in the original DEHP review, but do not report new information.

A 1-m segment of PCV tubing was used to measure DEHP release into polysorbate 80 solutions *(50)*. Physiological saline and distilled water solutions of polysorbate 80 resulted in greater DEHP release from tubing than did glucose solutions. Use of a flow rate of 90 mL/hour resulted in greater DEHP release than did 60 mL/hour. After 5 hours of infusion of 2 mg/mL polysorbate 80 at a rate of 90 mL/hour, the cumulative amount of DEHP recovered was 850 μg **[estimated from a graph]**. Recovery of DEHP was greater at 90 mL/hour than at 60 mL/hour even when the concentration of polysorbate 80 was increased 1.5-fold at the lower flow rate, suggesting to the authors that the amount of polysorbate passing through the tube segment was less important than the speed of the polysorbate micelles interacting with the walls of the tubing. **[The Expert Panel noted that the study is pertinent considering the very slow flow rate of TPN administered to neonates in NICU settings.]**

Hydrogenated castor oil, an emulsifier used in pharmaceuticals to increase solubility, was found to increase the release of DEHP from PVC tubing when given in distilled water or physiological saline *(51)*. Release appeared to increase linearly over time, reaching an approximate cumulative value of 776 μg DEHP after 4 hours. Sugar solutions (ribose, fructose, or glucose) containing HCO60 resulted in less DEHP release from tubing. DEHP levels increased with increasing HCO60 concentrations in all solutions.

Demore et al. *(52)* studied the release of DEHP from containers when the antineoplastic drug etoposide was stored. Etoposide was evaluated because it is prepared with the surfactant polysorbate 80, which is believed to release of DEHP from PVC containers. After 24 hours at room temperature in PVC containers, etoposide in saline contained 18–25 μg/mL DEHP, and etoposide in 5% dextrose contained 17–25 μg/mL DEHP. Etoposide in glass or polyolefin containers did not contain detectable levels of DEHP after similar time periods.

In Japan, DEHP was measured in a blood circuit system used to simulate hemodialysis and pump-oxygenation therapy using heparin-coated and uncoated PVC tubing *(53)*. In the hemodialysis system, the bovine blood used as the simulant had a baseline DEHP concentration of 249 ppb **[µg/L]**. After 4 hours of circulation, the DEHP concentration was 1718 ppb **[µg/L]**, a 7-fold increase. In the pump-oxygen system, PVC tubing with covalently bonded heparin coating resulted in DEHP levels approximately 50% lower than tubes with ionic-associated heparin coating or no coating at all. The DEHP daily dose for an 11 kg child exposed to 6 hours of pump-oxygenation therapy without heparin-coated tubes was estimated (using bovine blood) at 0.7 mg/kg bw/day and using heparin-coated tubing between 0.3 and 0.6 mg/kg bw/day. An adult exposure was estimated at approximately 0.3 mg/kg bw/day for uncoated tubing and between 0.16 and 0.3 mg/kg bw/day for heparin-coated tubings. These values were noted by the authors to be above the upper limit of the tolerable daily intake established by the Japanese Ministry of Health, Labor and Welfare. In the pump-oxygenation system, the authors estimated that 3–4% of DEHP was converted to MEHP. MEHP was also found to decrease with the use of covalently bonded heparin-coated PVC tubing, but concentrations increased over time. In the hemodialysis system, approximately 80 ppb **[µg/L]** MEHP was measured after 4 hours. The authors conclude that the use of PVC tubing for high-risk patients and for long-term therapy should be questioned. **[The Expert Panel noted that this study does not provide much new information with the exception that covalent and not ionic bonding of heparin to tubing is necessary to prevent significant leaching.]**

Mettang et al. *(54)* compared serum, urine, and dialysate levels of phthalate acid esters in 5 adult peritoneal dialysis patients before and 42 days after the use of plasticizer-free bags and tubing. Following the switch to plasticizer-free materials, significant changes included reductions in phthalic acid levels in serum and MEHP and phthalic acid levels in effluent dialysate. Serum concentrations of DEHP decreased non-significantly. There was no effect on levels of MEHP or 2-ethylhexanol in serum or phthalic acid in urine. The study authors concluded that peritoneal dialysis patients are likely exposed to sources of phthalates in addition to dialysis equipment.

In an effort to simulate exposure during respiratory therapy, Hill et al. *(55)* measured the concentrations of phthalates including DEHP in air after passage through PVC medical tubing. DEHP was detectable in an unspecified proportion of samples but was not above limits of quantification or was not present at concentrations demonstrably higher than background. The presence of 2-ethylhexanol was interpreted as due to DEHP breakdown. The authors concluded that for most adults, exposure from respiratory therapy is small compared to other exposures, but that sensitive populations, particularly those with allergies to plasticizers or with asthma, may be at "significant risk" from respiratory therapy exposures. **[The Expert Panel noted that findings are consistent with previous studies.]**

Platelet pheresis donors (n = 36) were evaluated for DEHP exposure by measuring serum DEHP concentrations before and after pheresis sessions of 38–89 minutes *(56)*. In 4 donors, additional serum samples were evaluated for up to 48 hours after the pheresis session. Median (range) serum DEHP increased from a baseline of 92.2 ng/mL (5.9–219.6 ng/mL) to 213.8 ng/mL (7.3–716.1 ng/mL). The authors estimated a median (range) DEHP dose of 6.46 (1.8–20.3) µg/kg bw. In the subjects with further serum monitoring, DEHP serum concentrations returned to baseline within 3 hours of the procedure. Serum triglyceride concentration was correlated with the relative increase in serum DEHP ($r^2 = 0.24$, $P = 0.03$).

Koch et al. *(57)* measured urinary concentrations of the DEHP metabolites MEHP, 5-hydroxy-MEHP, 5-oxo-MEHP, 5-cx-MEPP, and 2-cx-MMHP in a single platelet pheresis donor before and for 24 hours after the pheresis procedure. Over the 24-hour period, the total molar excretion of DEHP metabolites was 4.508 µmol. Urinary excretion ratios from different authors yielded estimates of DEHP dose of 2.6–4.0 mg or 31.6–48.1 µg/kg. The same authors evaluated 18 pheresis donors and 5 non-donor controls using 24-hour urine samples for measurement of MEHP, 5-hydroxy-MEHP, and 5-oxo-MEHP *(58)*. The first urine samples in the donors were collected just prior to the pheresis procedure. There were 6 donors who underwent plasma pheresis and 12 donors who underwent platelet pheresis (6 with a dual-needle continuous-flow technique and 6 with a single-needle discontinuous flow technique). Mean metabolite concentrations in urine shortly after pheresis were about twice as high for continuous flow techniques as for discontinuous flow techniques. Most metabolite excretion occurred during the first 5 hours after the pheresis procedure. Using metabolite excretion factors, DEHP doses were calculated as summarized in
Table 10. The authors compared the weight adjusted dose with the European Union tolerable daily intake values of 20–48 µg/kg bw. They suggested that the DEHP dose associated with plasma pheresis may not be elevated above background because the lipid-rich plasma removed by the procedure may contain most of the DEHP associated with exposure to the pheresis tubing.

Table 10. DEHP Dose with Pheresis Procedures

Procedure	Median dose (mg)	Mean dose (range), µg/kg bw
Platelet pheresis		
Continuous technique	2.10	32.1 (28.2–38.1)
Discontinuous technique	1.18	18.1 (14.3–23.8)
Platelet pheresis	0.37	5.7 (3.1–9.6)
Controls (no procedure)	0.41	6.2 (3.0–11.6)

From *(58)*.

The amount of DEHP retained by dialysis patients during a 4-hour dialysis treatment was estimated by measurement of DEHP blood levels in blood coming to the patient from the dialysis machine and in blood coming from the patient to the dialysis machine *(59)*. In all patients, a higher concentration of DEHP was present in blood entering the patient than in blood leaving the patient. The mean amount of DEHP retained by the patient after 4 hours of dialysis was 16.4 mg (range 3.6–59.6 mg). The authors used their data to construct a toxicokinetic model of DEHP transfer during dialysis. **[There was no discussion of MEHP infused during dialysis or created in vivo during dialysis.]**

1.6 Utility of Exposure Data

Estimates of DEHP exposures from medical devices have been made using simulated medical procedures with a variety of media. General population exposures have been estimated from urinary concentrations of DEHP metabolites. Other exposure estimates have been derived from measurements of DEHP or MEHP in food, blood, air, or environmental media.

1.7 Summary of Human Exposure Data

General population intake estimates for DEHP have been developed using probabilistic analysis (Clark, 2003 #337}. More than 90% of estimated daily DEHP intake in people over the age of 6 months is from food. Median estimates are given in Table 3 and range from 8.2 µg/kg bw/day in adults to 25.8 µg/kg bw/day in toddlers. Most estimates of general population exposures have been based on measurement of DEHP metabolites in urine, and a summary of those results is

included in Table 11. Which metabolite(s) are optimum for estimation of exposure is an issue that is currently being discussed, but 5-oxo- and 5-hydroxy-MEHP may be more accurate predictors of DEHP exposure due to their relatively high concentration in urine and their lack of susceptibility to contaminants in the sample collection process *(20, 24, 30)*. Estimates of general population exposures (95th percentile) using these urine metabolites are 65.0 μg/kg bw/day for men and 27.4 μg/kg bw/day for women *(20, 24)*. Not all investigators agree with the methods used to derive these estimates, and alternative estimates have been as much as 5-fold lower *(25)*.

Exposure to DEHP from medical devices is summarized in Table 12. DEHP extraction from iv tubing increases with increasing temperature and varies with the nature of the solution being administered. Infant ICU exposures were estimated by FDA *(2)* at 2.83 mg/kg bw/day. Blood transfusion is an important source of DEHP exposure, and FDA *(2)* estimated adult exposures at ≥4 mg/kg bw/day resulting from ECMO or multiple transfusions.

Estimated dietary daily intake of several plasticizers including DEHP involved with the preparation, packaging, and storage of food in 3 hospitals was calculated by Tsumura et al. (Tsumura, 2003 #121). The average daily DEHP intake was 160 μg/day **[3.2 μg/kg bw/day based on a 50 kg body weight]**.

Latini et al. (Latini, 2003 #149) found either DEHP or MEHP in 87.5% of 24 maternal plasma samples and 76% of 25 umbilical cord samples (1 set of twins). Mean ± SD DEHP concentrations were higher in cord plasma than in maternal plasma ($2.05 ± 1.47$ μg/mL compared to $1.15 ± 0.81$ μg/mL). MEHP concentrations in maternal and umbilical cord plasma were similar with mean ± SD values of $0.68 ± 0.85$ and $0.68 ± 1.03$ μg/mL for maternal and cord plasma, respectively.

Bouma et al. (Bouma, 2002 #187) measured DEHP released from 47 toys containing PVC after mixing with a saliva simulant. DEHP was found in 20 (43%) of the 47 toys at 30–45% by weight. Six toys exceeded the Dutch guidance release value (2.3 μg/min/10 cm^2) for children less than 1 year old.

Otake et al. *(36)* measured indoor air concentrations of common phthalates in 27 homes in the Tokyo metropolitan area. Indoor air concentrations of DEHP ranged from <0.001 to 3.13 μg/m^3. The mean ± SD concentration was $0.32 ± 0.6$ μg/m^3. DEHP levels were 100–1000 times higher than ambient outdoor concentrations reported in articles cited by the author: 2.0 ng/m^3 in Sweden and 16 ng/m^3 in Japan. Adibi et al. *(18)* measured phthalate diesters in 48-hour air monitoring samples collected by 30 pregnant women in New York and 30 pregnant women in Krakow, Poland. The median DEHP air concentrations (ranges) were: New York 0.22 (0.05–0.41) μg/m^3, Krakow 0.37 (0.08–1.1) μg/m^3.

Marttinen et al. *(40, 41)* found DEHP to be the most frequently encountered phthalate in sewage in Finland, with DEHP concentrations of 9–122 μg/L compared to < 5 μg/L for other phthalates. The highest concentration, 160–166 μg/L, was found in treated sewer sludge. In 4 sewage treatment plants, 80–96% of DEHP was removed, but the remaining DEHP accumulated in treated sludge.

Table 11. Summary of DEHP Metabolite Levels Measured in Human Urine

| Population | Urinary levels of metabolites | | | Reference |
	MEHP	5-hydroxy-MEHP	5-oxo-MEHP	
46 women (35–49 years old) from the U.S.; data presented as median (range)	7.3 (1.0–143.9) ng/mL or 6.4 (0.4–77.3) µg/g creatinine			Hoppin et al. *(14)*
2540 urine samples from adults and children older than 6 years from the US; data presented as geometric means	5.12 µg/L (5.19 µg/g creatinine) for children age 6–11, 3.75 µg/L (2.53 µg/g creatinine) for adolescents 12–19 years old, and 3.21 µg/L (3.03 µg/g creatinine) for adults 20 years and older			NHANES 1999–2000, presented by Silva et al. *(15)*
22782 urine samples from adults and children older than 6 years from the US; data presented as geometric means	4.41 µg/L (5.02 µg/g creatinine) for children age 6–11, 4.57 µg/L (3.53 µg/g creatinine) for adolescents 12–19 years old, and 4.20 µg/L (3.96 µg/g creatinine) for adults 20 years and older	33.6 µg/L (38.3 µg/g creatinine) for children age 6–11, 24.9 µg/L (19.2 µg/g creatinine) for adolescents 12–19 years old, and 18.1 µg/L (17.2 µg/g creatinine) for adults 20 years and older	23.3 µg/L (26.6 µg/g creatinine) for children age 6–11, 17.5 µg/L (13.5 µg/g creatinine) for adolescents 12–19 years old, and 12.0 µg/L (11.4 µg/g creatinine) for adults 20 years and older	NHANES 2001–2002 *(16)*
150 women age 20–73 yearsand 150 children age 11–12 years, presented as geometric mean	41.3 ± 50 µg/L in women and 13.3 ± 24 µg/L in children.			Koo and Lee *(27)*
19 children age 12–18 months in the US; data presented as a range	6.1 to 47.3 ng/mL			Brock et al. *(23)*
85 volunteers age 7–34 years in Germany, presented as median (range)	10.3 (<LOQ–177) µg/L or 9.2 (<LOQ–123) µg/g creatinine	46.8 (0.5– 818) µg/L or 40.2 (6.9–449) µg/g creatinine	36.5 (0.5–544) µg/L or 30.4 (6.4–262) µg/g creatinine	Koch et al. *(20, 24)*
A 61-year-old German male who consumed 48.10 mg **[641 µg/kg bw]** labeled DEHP;	3.63 mg/L	10.04 mg/L	6.34 mg/L	Koch et al. *(28)*

| Population | Urinary levels of metabolites | | | Reference |
	MEHP	5-hydroxy-MEHP	5-oxo-MEHP	
presented as peak levels				
62 urine samples from adults and children in the US; presented as median values	4.5 ng/mL	36 ng/mL	28 ng/mL	Barr et al. *(30)*
127 adults or children ≥6 years in the U.S., presented as median (5th–95th percentile)	< LOD (< LOD– 20.4) ng/mL	17.4 (< LOD–220) ng/mL	15.6 (< LOD–243) ng/mL	Kato et al. *(31)*
254 children age 3–14 years in Germany; presented as geometric mean (range)	7.91 (range 0.74– 226) µg/L	52.1 (1.86–2590) µg/L	39.9 (<0.5–1420) µg/L	Becker et al. *(22)*
36 children (age 2.6–6.5 years) in Germany; presented as median (range)	6.6 (1.5–18.3) µg/L or 8.7 (1.7–48.4) µg/g creatinine	49.6 (2.7–129) µg/L or 55.8 (15.4–258) µg/g creatinine	33.8 (2.2–90.6) µg/L or 38.3 (10.2–158) µg/g creatinine	Koch et al. *(21)*
19 adults in Germany; presented as median (range)	9.0 (2.6–43.1) µg/L or 8.6 (3.8–26.6) µg/g creatinine	32.1 (10.7–103) µg/L or 28.1 (10.9–63.6) µg/g creatinine	19.6 (4.9–55.1) or 17.2 (4.5–40.9) µg/g creatinine	Koch et al. *(21)* µg/L or
25 pregnant women in the US; presented as median (range)	4.60 (1.80–449) µg/g creatinine			Adibi et al. *(18)*

Table 12. Summary of DEHP Exposure Estimates from Medical Devices

Population	Medical device	Medium	Estimated DEHP exposure	Reference
Infants	IV tubing	Lipid-containing solutions used for parenteral nutrition	424.4 µg/mL over 24 hours; 5 mg/kg bw for a 2 kg infant receiving 25 mL solution	Loff et al. *(42)*
		Amino acid/glucose solution	0.83 µg/mL, 24 hours	
		1% propofol, continuous	6561 µg for a 2 kg infant	
		fentanyl solution 28.8 mL	132.5 µg for a 2 kg infant	
		midazolam 24 mL	26.4 µg for a 2 kg infant	
	Blood bag + tubing	Packed red blood cells, 20 mL	608 µg for a 2 kg infant	
		Platelet-rich plasma	928 µg for a 2 kg infant	
		Fresh frozen plasma	552–8108 µg for a 2 kg infant	
	IV tubing	Lipid-containing infusion solution, 27°C	422 µg/mL; 10 mg for a 2 kg infant receiving 24 mL	Loff et al. *(43)*
		Lipid-containing infusion solution, 33°C	540 µg/mL; 13 mg for a 2 kg infant receiving 24 mL	
Not specified	IV tubing	Hydrogenated castor oil in saline or water	775 µg after 4 hours	Hanawa et al. *(51)*
		Hydrogenated caster oil in sugar solutions	150 µg over 4 hours	

Population	Medical device	Medium	Estimated DEHP exposure	Reference
Not specified	PVC bags	Etoposide in polysorbate 80-containing solution	17–25 µg/mL after 24 hours	Demore et al. (52)
Children	Ethyl vinyl acetate bags with PVC connectors and tubing	Lipid-containing parenteral nutrition solution stored at 4ºC for 24 hours or 1 week	0.8–2 mg/day	Kambia et al. (44)
Not specified	PVC multi-layer iv tubing	Polysorbate solution	26–30 mg/mL after 2 hours; 62–70 mg/mL after 24 hours	Bourdeaux et al. (46)
Children and adults	Hemodialysis simulation	Bovine blood	1718 µg/L after 4 hours, compared to 249 µg/L at baseline. MEHP 80 µg/L after 4 hours. Estimated adult dose 0.067 mg/kg bw/day.	Haishima et al. (53)
	Pump-oxygenation simulation	Bovine blood	Estimated child's dose of 0.3–0.7 mg/kg bw/day; estimated adult dose of 0.16–0.3 mg/kg bw/day. MEHP 200–400 µg/L after 4 hours.	
Adults	Hemodialysis	Blood from 11 patients on therapy	Patients retained DEHP 16.4 mg (range 3.6–59.6 mg) after a 4-hour dialysis session	Dine et al. (59)
Adults	Platelet pheresis	Blood from 36 healthy donors	Median dose retained after pheresis session was 6.46 µg/kg bw (range 1.8–20.3 µg/kg bw)	Buchta et al. (56)
Adults	Platelet pheresis	Blood from 12 healthy donors	Median dose retained after pheresis session was 18.1–32.3 µg/kg bw (range 14.3–38.1 µg/kg bw)	Koch et al. (4)
Infants	All ICU exposures	Aggregate exposures	2.83 mg/kg bw/day	FDA (2)
Adults	Medical treatment	Aggregate exposures	0.005–8.5 mg/kg bw/day	FDA (2)
Adults	ECMO, multiple transfusions	Blood	≥ 4 mg/kg bw/day	
Not specified	Respirator PVC tubing	Air	Below limits of quantification	Hill et al. (55)

2.0 GENERAL TOXICOLOGY AND BIOLOGIC EFFECTS

Section 2 of this report contains summaries of toxicokinetics, general toxicity, or carcinogenicity studies that may be especially relevant to the interpretation of developmental and reproductive effects associated with DEHP exposure.

2.1 Toxicokinetics

The metabolism of DEHP in humans is discussed in Section 1 with respect to the use of urinary metabolite concentrations in the estimation of DEHP exposures. Metabolism has also been summarized by Koch et al. *(4)*. By 24 hours after an oral DEHP dose, about 70% appears in the urine as 5 major metabolites (see Table 6). According to this review, metabolism is similar after iv exposure to DEHP.

Toxicokinetic studies using radiolabeled DEHP by gavage in pregnant and non-pregnant female Wistar rats and CD-1 mice appeared in unpublished reports sponsored by the European Council for Plasticizer and Intermediates *(60, 61, 62, 63, 64)*. Determinations were made after single doses of 200 or 1000 mg/kg bw and after 5 daily doses at these levels. Results are summarized in Table 13.

Table 13. Toxicokinetic Parameters in Pregnant (GD 6) and Non-pregnant Female Rats and Mice Given Oral Radiolabeled DEHP

Model	C_{max} (nmol-eq/mL)	AUC_{0-48} (nmol-eq-h/mL)	$t_{1/2}$ (hours)
Single 200-mg/kg bw dose			
Non-pregnant rat	64	1426	7.1
Pregnant rat	58 (32.1/36.4)[a]	983 (217/511)	7.8 (82.1/5.9)
Non-pregnant mouse	154	2069	7.1
Pregnant mouse	91 (28/84)	1078 (171/816)	7.3 (-/4.0)
Single 1000-mg/kg bw dose			
Non-pregnant rat	353	5825	10.2
Pregnant rat	249 (90.8/146.3)	6254 (1180/2445)	5.5 (-/14.0)
Non-pregnant mouse	1339	6838	10.3
Pregnant mouse	227 (103/215)	6745 (1107/3526)	9.7 (-/14.2)
Repeated 200-mg/kg bw dose			
Non-pregnant rat	77	1007	8.7
Pregnant rat	98	1606	10.3
Non-pregnant mouse	197	2252	7.1
Pregnant mouse	90	1083	7.3
Repeated 1000-mg/kg bw dose			
Non-pregnant rat	405	6398	13.5
Pregnant rat	518	7410	6.6
Non-pregnant mouse	396	5672	7.9
Pregnant mouse	551	4890	11.4

[a]Figures are given for total radioactivity with (DEHP/MEHP) in parentheses, as determined by gas chromatography (GC). C_{max} = maximum concentration; t_{max} = time to maximum concentration; $t_{1/2}$ = half-life; AUC = area under the concentration–time curve.

An unpublished report from Mitsubishi Chemical Safety Institute, Ltd. *(65)* described a 65-week oral-dose toxicity study of DEHP in marmosets (discussed in section 4.2.2.2) and included a toxicokinetic study. **[Some data from this study were published in abstract *(66)*.]** The study was sponsored by the Japan Plasticizer Industry Association. Ring-labeled ^{14}C-DEHP (99.6%

purity) in corn oil was given to 3 groups of marmosets. The first group was treated at 3 months of age. The second group was treated at 18 months of age. The third group was treated for 65 weeks from 3 months of age with unlabeled DEHP and studied at 18 months of age. There were 3 animals of each sex in each treatment group. Treatments were by gavage at dose levels of 100 or 2500 mg/kg bw. Blood samples were collected 1, 2, 4, 8, 12, 24, 48, 72, 120, and 168 hours after dosing. Spontaneous urine and feces were collected for radioactivity determination. At least 2 weeks after the kinetic studies, animals were dosed again and tissues collected 2 hours later for determination of radioactivity. Radioactivity determination was by liquid scintillation counting. Toxicokinetic parameters are shown in Table 14. Reproductive organ radioactivity contents 2 hours after dosing are shown in Table 15. The authors found the highest level of radiation in the kidneys after a single oral dose, and considered that high radioactivity levels in the prostate and seminal vesicles of some animals may have been due to urine contamination. Repeated dosing for 65 weeks did not appear to alter the distribution of DEHP in 18-month-old animals. The authors called particular attention to the small amount of label distributed to the testis and postulated that differences in access of DEHP metabolites to the testis may explain a lack of testicular toxicity in marmosets compared to rodents, in which large amounts of MEHP are distributed to the testis after DEHP treatment.

Table 14. Toxicokinetic Parameters after Oral Dosing of Marmosets at Age 3 and 18 Months

Dose group	C_{max} (µg eq/mL)	t_{max} (hour)	$t_{1/2}$ (hour)	AUC (µg eq-hour/mL)	AUC/dose (hour-kg/L)
3 Month olds					
100 mg/kg bw					
Male	6.86±4.86	4.0±3.5	8.0±4.0	37.4±19.3	0.374±0.193
Female	17.08±10.69	1.3±0.6	6.0±3.5	78.7±67.2	0.787±0.672
2500 mg/kg bw					
Male	36.00±37.47	10.0±12.2	21.3±23.1	270.2±194.5	0.108±0.078
Female	66.00±22.34	4.0±0.0	8.0±0.0	347.7±66.5	0.139±0.027
18 Month olds					
100 mg/kg bw					
Male	13.53±6.07	2.3±1.5	5.3±2.3	99.0±57.4	0.990±0.574
Female	19.49±16.71	1.0±0.0	4.7±3.1	150.8±137.8	1.508±1.378
2500 mg/kg bw					
Male	50.00±39.23	1.0±0.0	2.7±1.2	444.7±197.8	0.178±0.079
Female	62.67±38.73	1.3±0.6	4.7±3.1	952.8±1093.3	0.381±0.437
18 Month olds after 65 weeks pretreatment					
100 mg/kg bw					
Male	14.77±17.04	2.3±1.5	5.3±2.3	83.0±104.9	0.830±1.049
Female	4.81±3.61	2.3±1.5	6.7±4.6	48.2±46.0	0.382±0.460
2500 mg/kg bw					
Male	32.33±6.43	1.0±0.0	2.7±1.2	153.7±18.9	0.061±0.008
Female	4.33±2.31	1.0±0.0	2.7±1.2	11.5±10.3	0.004±0.004

Data are mean ± SD, n = 3 animals/sex/group. From *(65)*.

Table 15. Reproductive Organ Radioactivity Content 2 Hours after Oral Dosing Marmosets with Radiolabeled DEHP

Dose group	Concentration (µg eq/mL or µg eq /mg)			Organ/plasma ratio	Distribution (% of dose)
	Male plasma	Female plasma	Organ		
3 Month olds					
100 mg/kg bw	26.54±35.56	33.55±29.22			
Testis			5.47±7.35	0.21	0.002±0.003
Epididymis			10.25±13.28	0.39	0.002±0.002
Prostate			13.04±8.79	0.49	0.001±0.002
Seminal Vesicle			8.03±4.57	0.30	0.001±0.001
Ovary			4.84±4.33	0.14	0.000±0.000
Uterus			11.28±13.16	0.24	0.003±0.004
2500 mg/kg bw	45.51±31.47	45.50±45.92			
Testis			10.52±4.63	0.23	0.000±0.000
Epididymis			14.09±2.54	0.31	0.000±0.000
Prostate			34.22±30.49	0.75	0.000±0.000
Seminal vesicle			23.14±15.18	0.51	0.000±0.000
Ovary			not detected	–	–
Uterus			8.91±9.49	0.20	0.000±0.000
18 Month olds					
100 mg/kg bw	9.01±9.97	16.88±13.48			
Testis			0.83±0.81	0.09	0.003±0.004
Epididymis			1.97±1.29	0.22	0.001±0.001
Prostate			4.59±4.52	0.51	0.002±0.002
Seminal vesicle			16.26±21.74	1.80	0.005±0.006
Ovary			5.93±4.25	0.35	0.004±0.004
Uterus			3.79±2.64	0.22	0.002±0.002
2500 mg/kg bw	65.48±95.58	123.74±33.78			
Testis			8.47±11.95	0.13	0.001±0.001
Epididymis			15.98±20.15	0.24	0.000±0.001
Prostate			10.64±14.53	0.16	0.000±0.000
Seminal vesicle			13.84±15.79	0.21	0.000±0.001
Ovary			36.11±11.39	0.29	0.001±.000
Uterus			33.52±12.62	0.27	0.001±0.001
18 Month olds after 65 weeks pretreatment					
100 mg/kg bw	29.92±6.61	47.28[a]			
Testis			3.03±0.97	0.10	0.011±0.004
Epididymis			9.79±6.91	0.33	0.008±0.008
Prostate			7.34±3.34	0.25	0.002±0.002
Seminal vesicle			12.60±11.95	0.42	0.004±0.004
Ovary			14.12[a]	0.30	0.006[a]
Uterus			9.24[a]	0.20	0.004[a]
2500 mg/kg bw	102.78±81.44	41.70±29.53			
Testis			12.40±9.07	0.12	0.002±0.002
Epididymis			27.98±20.66	0.27	0.001±0.001
Prostate			20.38±14.74	0.20	0.000±0.000
Seminal vesicle			23.73±18.80	0.23	0.000±0.000
Ovary			13.18±8.51	0.32	0.000±0.000
Uterus			9.97±6.12	0.24	0.000±0.000

Data are mean ± SD, n = 3 animals/sex/group. [a]There were only 2 females in this group.
From (65).

Kessler et al. *(67)*, sponsored in part by the American Chemistry Council, compared blood levels of DEHP and MEHP in pregnant and non-pregnant Sprague-Dawley rats and marmosets **[strain not indicated]** in a Good Laboratory Practice (GLP) study. DEHP or deuterium-labeled DEHP were dissolved in an aqueous Tween 80/Methocel/saccharose solution that was fed to marmosets through a syringe following their first meal and administered to Sprague-Dawley rats by gavage. In most cases, the deuterated-DEHP was administered in at least 1 dose group on the days that time-course experiments were conducted in order to differentiate between background DEHP and MEHP. Non-pregnant female rats (n = 3–4 group) were dosed with 30, 500, or 1000 mg/kg bw. Rats in the 500 mg/kg bw group were dosed for 7 days and time-course experiments were conducted on study days 1, 4, and 7. Pregnant rats were dosed with 30 or 500 mg/kg bw/DEHP on gestation day (GD) 14–20, and concentration time courses were determined on GD 14 and 19 **[of a 21–22 day gestation]**. In rats, blood samples were collected over a 24–48 hour period following dosing. Non-pregnant marmosets (n = 8/dose) were treated with 30 or 500 mg/kg bw/day DEHP for 29 days; concentration time courses were determined on treatment days 1 and 29. Pregnant marmosets (n = 4/dose) were dosed with 30 or 500 mg DEHP/kg bw/day on GD 96–125; concentration time courses were determined GD 96, 103, 117, and 124 **[of a 140–148 day gestation]**. On days when concentration-time courses were determined, blood samples were drawn over 15 hours following exposure of non-pregnant marmosets and 8 hours following exposure of pregnant marmosets. Because blood could be drawn only once per week from the arm vein of the marmosets, each time point of the blood sampling curve was represented by 1 animal. Blood levels of DEHP and MEHP were determined by GC/MS.

Area under the concentration–time curve (AUC) values determined in rat studies are listed in Table 16. Authors concluded that concentration time courses were similar in pregnant and non-pregnant rats and that repeated dosing had no marked effects on kinetics in either group of rats. In both groups of rats, MEHP blood AUCs were about 2 orders of magnitude higher than DEHP blood AUCs. For the non-pregnant rats, maximum concentrations for DEHP were obtained at about 1 hour following dosing; maximum concentrations of MEHP following dosing were reached at 30 minutes in the 30 mg/kg bw group, 2 hours in the 500 mg/kg bw group, and 4 hours in the 1000 mg/kg bw group. Based on normalized AUCs that were not dose dependent, the authors concluded that kinetics were linear for DEHP. The authors concluded that kinetics for MEHP were saturated based on AUC values and increased time to reach maximum concentration at higher doses.

AUC values for marmosets are listed in Table 17. Concentration time courses were similar in pregnant and non-pregnant marmosets with the exception that MEHP values in the 500 mg/kg bw DEHP group were lower compared to the non-pregnant animals at GD 103 and beyond. In the non-pregnant marmosets, DEHP concentrations peaked at 2 hours following dosing; MEHP concentrations returned to starting levels within 15 hours following dosing. MEHP AUCs in pregnant and non-pregnant marmosets were more than an order of magnitude higher than DEHP AUCs and were independent of dose.

In a comparison of species differences, maximum concentrations of MEHP in rats were an average of 3.2 times higher (range 1.3–7.5) than those of marmosets. MEHP AUCs were an average of 7.3 times higher (range 2.6–15.6) in rats compared to marmosets. Based on maternal blood levels, the study authors concluded that the MEHP burden in marmoset fetuses is lower than in rat fetuses. **[The Expert Panel notes that the burden to rats compred to marmosets was not determined.]**

Table 16. Normalized Areas under the Concentration–time Curve for Blood DEHP and MEHP in Rats Treated with DEHP

DEHP dose (mg/kg bw/day)	Treatment day	DEHP AUC (nmol-h/mL per mmol DEHP/kg)[a]	MEHP AUC (nmol-h/mL per mmol DEHP/kg)[a]
		Non-pregant rats	
30	1	Not determined	695±113
500	1	5.9±3.1	1058±60
	4	7.1±3.1	1104±423
	7	4.7±0.7	1237±636
1000[b]	1	8.4±4.4	1756±838
		Pregnant rats	
30[c]	1 (GD 14)	8.5±3.6	606±77
	6 (GD 19)	21.0±7.9	646±42
500[c]	1 (GD 14)	10.0±5.4	1537±158
	6 (GD 19)	12.7±6.3	1106±230

[a]Total normalized AUC presented as mean ± SD.
[b]Deuterated DEHP administered to 2 of 4 animals.
[c]Deuterated DEHP administered all animals.
From Kessler et al. *(67)*.

Table 17. Areas under the Concentration–time Curves for Blood DEHP and MEHP in Marmosets Treated with DEHP

DEHP dose (mg/kg bw/day)	Treatment day	DEHP AUC (nmol-h/mL per mmol DEHP/kg)[a]	MEHP AUC (nmol-h/mL per mmol DEHP/kg)[b]	MEHP AUC (nmol-h/mL per mmol DEHP/kg)[a]
		Non-pregnant marmosets		
30[c]	1	8.9	172	181
	29	6.5	112	118
500	1	1.2[d]	100[d]	Not determined
	29	2.5	123	130
		Pregnant marmosets		
30[c]	1 (GD 96)	5.6	178	Not determined
	8 (GD 103)	5.2	258	Not determined
	22 (GD 117)	3.5	154	Not determined
	29 (GD 124)	6.4	245	Not determined
500	1 (GD 96)	12.3	170	Not determined
	8 (GD 103)	4.1	31	Not determined
	22 (GD 117)	2.8	63	Not determined
	29 (GD 124)	3.4	71	Not determined

[a]Total normalized AUC (unless otherwise indicated).
[b]Normalized AUC (up to 8 hours unless otherwise indicated).
[c]Deuterated DEHP administered to all animals.
[d]Normalized AUC up to 6 hours.
From Kessler et al. *(67)*.

Studies on urinary metabolites of DEHP in humans have been addressed in Section 1 because these studies have been focused on the use of metabolites in estimating DEHP exposure.

2.2 General Toxicity and Carcinogenicity

Conclusions in recent reviews by the FDA, Health Canada, and the European Commission are summarized in Table 18.

Stroheker et al. *(68)* evaluated the anti-androgenic activity of DEHP in a modified Hershberger assay using Wistar rats. Male offspring were weaned and randomized by weight at 20 days of age **[day of birth not defined]**. On the following day, the animals were castrated and allowed to recover for 1 week. DEHP (>99% purity) in corn oil was given by gavage for 10 days at 0, 200, 400, 600, 800, or 1000 mg/kg bw/day in the first experiment and 0, 4, 20, or 100 mg/kg bw/day in the second experiment (n = 8/treatment group). In both experiments, testosterone propionate 0.4 mg/kg bw/day was given subcutaneously (sc) on the same days as the DEHP treatments. The animals were weighed and killed 24 hours after the last treatment and relative weights were determined for the seminal vesicles, prostate, and bulbocavernosus/levator ani muscles. As expected, testosterone propionate treatment produced a significant increase in the relative weight of all accessory sex organs compared to vehicle-treated control. A significant impairment of the testosterone propionate-induced organ weight increase occurred with DEHP treatment beginning at 100 mg/kg bw/day for the bulbocavernosus/levator ani muscles, 200 mg/kg bw/day for the prostate, and 400 mg/kg bw/day for the seminal vesicles. The authors concluded that DEHP treatment has anti-androgenic effects but does not inhibit 5α-reductase because bulbocavernosus/levator ani muscles, the most sensitive organs, are only testosterone-responsive, whereas prostate is only dihydrotestosterone-responsive, and seminal vesicles are responsive to both androgens. **[The Expert Panel noted that it is not clear if testosterone propionate data were combined or compared separately for the two blocks. The lack of dose-response was noted.]**

In the same report, Stroheker et al. *(68)* evaluated DEHP, MEHP, 5-oxo-MEHP, and 5-hydroxy-MEHP in an androgen receptor-positive breast cancer cell line stably transfected with a luciferase reporter gene. The cell line showed an 81% decrease in dihydrotestosterone-induced luciferase activity after exposure to the positive control nilutamide, an androgen receptor antagonist, at 10^{-6} M. DEHP and MEHP were added to cultures at log unit concentrations ranging from 10^{-10} to 10^{-5} M. The secondary DEHP metabolites 5-oxo- and 5-hydroxy-MEHP were added at 10^{-10} to 10^{-8} M, limited by solubility or cytotoxicity. **[The method for evaluating cytotoxicity was not described.]** There was no inhibition of dihydrotestosterone stimulation of luciferase activity at any tested concentration of DEHP or MEHP. Both 5-oxo- and 5-hydroxy-MEHP inhibited luciferase activity at all tested concentrations to 40–70% of control levels **[estimated from a graph]**. The authors concluded that although in vivo anti-androgenic activity of DEHP could be indirect, due to increased catabolism of testosterone, it might alternatively be due to the anti-androgenic activity of the 5-oxo- and 5-hydroxy-MEHP metabolites.

Table 18. DEHP Conclusions by US, Canadian, and European Agencies

Topics	Agency			
	FDA *(2)*	ATSDR *(69)*	Health Canada *(70)*	European Commission *(1)*
Most sensitive target organ	Testis	Testis	Testis and conceptus	Testis
Other possible targets of toxicity	One study suggested that DEHP could contribute to hyaline membrane disease in mechanically ventilated children. Factors such as poor bowel perfusion more likely contribute to necrotizing enterocolitis in newborns than DEHP.	Although confounded, there is some evidence suggesting that DEHP released from PVC tubing during respiratory ventilation can cause lung disorders in children.		Suspicions about development of polycystic kidney disease in patients undergoing hemodialysis have not been confirmed by clinical evidence. Causation cannot be determined for the role of DEHP in pathological lung effects in ventilated preterm infants. Evidence suggests that DEHP is not a causative agent of hepatoblastoma.
Genetic toxicity		The weight of evidence indicates that DEHP is not genotoxic.		
Cancer		Mechanisms of liver cancer in rats and mice are not relevant to humans.	Concurs with IARC conclusion that mechanisms of liver tumors in rodents are not relevant to humans.	There are no concerns about carcinogenicity in humans, based on animal studies.
Sensitive populations	Children receiving some medical treatments may receive a higher dose on a mg/kg bw basis than adults. Compared to adults, children may absorb greater amounts of DEHP due to greater intestinal permeability, may more effectively convert DEHP to MEHP (the toxic metabolite) due to higher levels of intestinal lipases, and may less	Infants have higher levels of gastric lipases and may be more able to convert DEHP to MEHP. Permeability of blood-testis barrier is higher in children. There appear to be few indications of biological polymorphisms that increase sensitivity. Younger animals appear to	Populations at highest risk of DEHP toxicity include newborns, infants toddlers, and children with critical illness. Populations with unknown risk of toxicity include breast fed children, the fetus, and pre-pubescent males.	There is evidence of greater DEHP sensitivity in immature compared to mature animals.

Topics	Agency			
	FDA *(2)*	ATSDR *(69)*	Health Canada *(70)*	European Commission *(1)*
	effectively excrete MEHP due to reduced glucuronidation. Children may be more pharmacodynamically sensitive to DEHP than adults (e.g., increased permeability of the blood-testis barrier). DEHP may exacerbate zinc and vitamin E deficiencies, which are not uncommon in preterm infants. There are polymorphisms in genes coding for pancreatic lipase. There are polymorphisms in several UDP-glycuronyltransferase genes.	be more sensitive to DEHP-induced toxicity that older animals.		
Species differences	Liver effects mediated through PPARα do not appear relevant to humans.	Liver effects mediated through PPARα do not appear relevant to humans. DEHP hydrolysis rates are highest in mouse > rat >guinea pig, > hamster > humans and primates. Primates are more efficient at glucuronidating metabolites but less effective at oxidizing metabolites than rodents.		It is believed that DEHP toxicity in rodents is mediated through the PPARα receptor, which is less relevant in humans; however, there is also evidence that PPARα-independent toxicity also occurs. Mechanisms of adverse effects in rodents do not appear to be of great significance in non-human primates; evidence that those mechanisms apply to humans is lacking.
Acceptable limits	TI (oral) = 0.04 mg/kg bw/day. TI (parenteral) = 0.6 mg/kg bw/day.	MRL = 0.1 mg/kg bw/day for oral exposures of intermediate duration (15–364 days). MRL = 0.06 mg/kg bw/day for oral exposures of chronic		No Tolerable Intake Value can be recommended regarding use of DEHP in medical devices.

Topics	Agency			
	FDA *(2)*	ATSDR *(69)*	Health Canada *(70)*	European Commission *(1)*
		duration (≥ 365 days).		
Situations where DEHP exposures may be of toxicological concern	". . . children undergoing certain medical procedures may represent a population at increased risk for the effects of DEHP." Medical procedures of possible concern include: TPN in infants and pregnant women, enteral nutrition; exchange transfusions in infants; ECMO in infants and adults; aggregate exposures of neonates in NICU -- cardiopulmonary by-pass surgery may lead to high exposure but exposures vary widely depending on use of heparin-coated tubing.		Subpopulations at greatest risk: ECMO patients, cardiopulmonary by-pass patients, infants, and children receiving exchange transfusions, patients receiving some iv therapies such as TPN and lipophilic drug formulations. Subpopulations with possible but undetermined risk: trauma patients receiving multiple blood transfusions, hemodialysis patients, patients receiving oxygen therapy.	Premature infants are a particular risk group because they can be exposed to high DEHP concentrations through blood transfusions, ECMO, and respiratory therapy.
Situations not likely to result in toxicological concern	Infusion (iv) of crystalloid fluids and drugs. TPN in adults. Blood transfusions. Hemodialysis and peritoneal dialysis.	Ambient levels in environment.	DEHP levels do not pose a danger to the environment on which human life is dependent.	

ECMO extra corporeal membrane oxygenation; MRL minimal risk level; TI tolerable intake.

Voss et al. *(71)* administered DEHP (>99% purity) in the diet to male Sprague-Dawley rats beginning at an age of 90–110 days and continuing for the entire lifetime of the animals (up to 159 weeks). DEHP was administered in feed at 0, 600, 1897, and 6000 mg/kg diet, given in 5 g feed/100 g bw/day 6 days/week. On the 7th day of the week, animals received DEHP-free feed after their DEHP-treated feed had been completely consumed. DEHP dose levels were 0 (n=390), 30 (n=180), 95 (n=100), and 300 (n=60) mg/kg bw/day **[6 days/week unless residual treated feed was consumed on the 7th day. Daily feed consumption was not reported.]** The number of animals in each group was chosen based on anticipated tumor incidence, with larger numbers of animals in groups expected to have a lower incidence of tumors. Animals were killed when moribund if they did not die spontaneously, and all animals were necropsied after death. Brain, liver, adrenals, testes, thyroid, lungs, spleen, and macroscopic lesions were fixed in 7% formalin, sectioned in paraffin, and examined by light microscopy after staining with hematoxylin and eosin. Livers were weighed, and liver slices were fixed in Carnoy fluid. In addition to hematoxylin and eosin stained sections, liver evaluation included treatment with periodic acid-Schiff with orange G and iron hematoxylin counterstaining. Statistical evaluation was performed with Kruskal-Wallis and chi-squared tests.

The animals fed DEHP in all dose groups experienced a transient absolute weight reduction compared to control animals about 300 days after the beginning of the experiment but weights were comparable thereafter. The authors described a dose-dependent increase in liver weight, reaching 108% of control values in the highest dose group but indicated that liver weights were not statistically different from controls. There was no effect of DEHP treatment on survival time of the animals. The proportion of animals with malignant and benign tumors, overall, was not affected by treatment; however, detailed evaluation of livers of the sacrificed animals showed a 29.0% incidence of all neoplasms in the highest DEHP dose group compared to 9.0% of control animals ($P = 0.005$). Although the lower 2 DEHP dose groups did not have a statistically increased incidence of hepatic neoplasms on pair-wise comparison with the control, trend testing showed a significant trend over the dose ranges ($P = 0.001$).

Leydig cell tumors occurred in 28.3% of animals in the highest dose group compared to 16.4% of control animals ($P = 0.038$), and a dose-related trend was identified in Leydig cell tumors over the dose range ($P = 0.019$). The association of DEHP treatment with Leydig cell tumors extended to analysis of unilateral, bilateral, and multifocal unilateral tumors. When the lifetimes of the animals were divided into 3 periods (0–750, 750–950, and 950–1250 days), the associations between total and unilateral Leydig cell tumors and DEHP dose level were most evident during the middle period. Bilateral and multifocal unilateral tumors showed dose-related DEHP increases during the third period. The authors postulated that the DEHP-associated increase in Leydig cell tumors might be due to an increase in gonadotropin production secondary to decreased testosterone synthesis or increased testosterone aromatization.

2.3 Summary of General Toxicology and Biologic Effects

2.3.1 Toxicokinetics

An unpublished report from Mitsubishi Chemical Safety Institute, Ltd. *(65)* included a toxicokinetic study in marmosets at 3 and 18 months of age. The 18-month-old animals included a group that had been pretreated with radiolabeled DEHP for 65 weeks and a treatment-naïve group. Blood was drawn at intervals during the week following a single dose of 100 or 2500 mg/kg bw. Two weeks later, an additional oral dose was given and tissues were sampled 2 hours later. The report presented radioactivity contents, expressed as µg equivalents, without characterization of unchanged DEHP or DEHP metabolites. There was no apparent effect of chronic DEHP treatment on toxicokinetic parameters or organ distribution at 18 months of age. Concentrations of radiolabel in testis were 9–23% of plasma concentrations, and the authors

remarked that the small amount of DEHP and metabolites distributed to the marmoset testis might explain the lack of testicular toxicity noted by them in a 65-week feeding study (discussed in Section 4.2.2.2).

A GLP study compared blood levels of DEHP and MEHP in pregnant and non-pregnant rats and marmosets dosed with 30 or 500 mg/kg bw/day DEHP *(67)*. Authors concluded that concentration time courses were similar in pregnant rats receiving both dose levels by gavage on GD 14–20 and non-pregnant rats given 500 mg/kg bw/day for 7 days; repeated dosing had no marked effects on kinetics in either group of rats. In both groups of rats, MEHP AUCs were about 2 orders of magnitude higher than DEHP AUCs. The authors concluded that kinetics for DEHP were linear, while kinetics for MEHP were saturated. In marmosets fed 30 or 500 mg/kg bw/day DEHP through a syringe, concentration time courses (based on 1 marmoset per time point) were similar in pregnant animals dosed on GD 96–125 and non-pregnant animals dosed for 29 days; the exception was that MEHP values in the 500 mg/kg bw DEHP group were lower compared to the non-pregnant animals on GD 103 and beyond. MEHP AUCs in pregnant and non-pregnant marmosets were more than an order of magnitude higher than DEHP AUCs and were independent of dose. In a comparison of species differences, maximum blood concentrations of MEHP in rats were an average of 3.2 times higher (range 1.3–7.5) than those of marmosets. MEHP AUCs were an average of 7.3 times higher (range 2.6–15.6) in rats compared to marmosets.

2.3.2 General Toxicity and Carcinogenicity

The anti-androgenic activities of DEHP and some of its metabolites were evaluated by Stroheker et al. *(68)*. Treatment of castrated 21-day-old Wistar rats with DEHP prevented an increase in accessory sex-organ weight after testosterone propionate. The bulbocavernosus/levator ani muscles were the most sensitive to this DEHP effect, and the prostate was the least sensitive, leading the authors to conclude that DEHP did not inhibit 5α-reductase. Evaluation of DEHP, MEHP, and the secondary DEHP metabolites, 5-oxo- and 5-hydroxy-MEHP, in an androgen receptor-positive cell line showed no antagonism by DEHP or MEHP of the activity of the androgen receptor agonist dihydrotestosterone. Both 5-oxo- and 5-hydroxy-MEHP showed significant dihydrotestosterone antagonism. The authors concluded that the in vivo anti-androgenic effects of DEHP could be mediated through the secondary metabolites.

Voss et al. *(71)* administered DEHP at 0, 600, 1897, and 6000 mg/kg diet in the diet to male Sprague-Dawley rats beginning at an age of 90–110 days and continuing for the entire lifetime of the animals. DEHP dose levels were 0, 30, 95, and 300 mg/kg bw/day **[6 days/week unless residual treated feed was consumed on the 7th day]**. The animals fed DEHP experienced a transient weight reduction compared to control animals about 300 days after the beginning of the experiment, but weights were comparable thereafter. The authors described a dose-dependent increase in liver weight, reaching 108% of control values in the highest dose group, but indicated that liver weights were not statistically different from controls. There was no effect of DEHP treatment on survival time of the animals. The proportion of animals with malignant and benign tumors, overall, was not affected by treatment; however, detailed evaluation of livers of the sacrificed animals showed a 29.0% incidence of all neoplasms in the highest DEHP dose group compared to 9.0% of control animals ($P = 0.005$). Although the lower 2 DEHP dose groups did not have a statistically increased incidence of hepatic neoplasms on pair-wise comparison with the control, trend testing showed a significant trend over the dose ranges ($P = 0.001$). Leydig cell tumors occurred in 28.3% of animals in the highest dose group compared to 16.4% of control animals ($P = 0.038$), and a dose-related trend was identified in Leydig cell tumors over the dose range ($P = 0.019$). The authors postulated that the DEHP-associated increase in Leydig cell tumors might be due to an increase in gonadotropin production secondary to decreased testosterone synthesis or increased testosterone aromatization.

3.0 DEVELOPMENTAL TOXICITY DATA

3.1 Human Data

Latini et al. *(72)*, funding not indicated, conducted a study to examine the effects of prenatal exposure to DEHP and MEHP. Cord blood samples were collected from 84 consecutive newborns (including 2 twins) delivered at an Italian hospital. Ages of mothers ranged from 18 to 42 years. DEHP and MEHP levels were measured in cord blood serum by HPLC. Glass equipment was used in sample preparation and analyses to avoid phthalate contamination. Analyses were conducted to determine possible relationships between phthalate exposure and adverse neonatal outcomes. Relationships between phthalates in cord blood and outcomes in infants were assessed by Fisher exact test and unpaired *t*-tests. Significance levels for multiple *t*-tests were Bonferroni corrected. Multivariable logistic regression models were used to evaluate significant differences in univariate analyses and effects from potential confounders. DEHP and/or MEHP were detected in 74 of 84 cord blood samples. Mean cord blood serum concentrations were 1.19 μg/mL (range 0–4.71 μg/mL) for DEHP and 0.52 μg/mL (0–2.94 μg/mL) for MEHP. Mean gestational age was significantly lower in MEHP-positive neonates (38.16 ± 2.34 [SD] weeks) versus MEHP-negative neonates (39.35 ± 1.35 weeks) (*P* = 0.033). Logistic regression analyses demonstrated a positive correlation between lack of MEHP in cord blood and gestational age at delivery (OR 1.50, 95% CI 1.013–2.21). **[The significant correlation was expressed using a chi-squared statistic. It is not clear how an odds ratio was derived.]** There were no significant associations between DEHP or MEHP concentrations and infant sex, delivery mode, maternal smoking, premature membrane rupture, cord loops, neonatal jaundice, small infant size, birth weight, 1- or 5-minute Apgar scores, or maternal age. **[With the exception of birth weight, data were not shown for these endpoints.]**

The study authors concluded that their study demonstrated the presence of DEHP/MEHP in most newborns, and that phthalate exposure is associated with shorter pregnancy duration.

Strengths/Weaknesses: Use of cord blood, which reflects infant exposure in utero, is a strength as is the use of consecutive births at same hospital. The collection of blood in glass containers can be expected to have reduced the likelihood of contamination, although contamination with phthalates during analysis is still possible. Outcome assessment determination blind to exposure status is a strength. Weaknesses include the possible existence of influential outliers. In addition, some aspects of the data analyses were unclear.

Utility (Adequacy) for CERHR Evaluation Process: This study is moderately useful in the evaluation process.

Swan et al. *(73)*, supported by EPA, NIH, and the state of Iowa, evaluated anogenital distance in children and maternal urinary phthalate monoester concentrations. Pregnant women in 1 of 3 US cities were recruited as part of a larger study. Urine samples were collected from women at a mean gestational age of 28.3 weeks **[variance not reported]**. There were 85 participants whose urine samples were used for this study from among 172 women who were eligible for this part of the study and who bore sons. Exclusions included mother-son dyads with incomplete information, lack of consent for genital examination, and child age greater than 18 months **[considered to make anogenital distance measurement unreliable due to movement]**. The urine analytes included metabolites of DEHP (MEHP, 5-oxo-MEHP, and 5 hydroxy-MEHP) and monobutyl, monobenzyl, mono-3-carboxypropyl, monoethyl, mono-isobutyl, and monomethyl phthalate. Analysis was performed by HPLC-tandem MS after enzymatic deconjugation. Infant evaluation

included height, weight, head circumference, skin-fold thickness, anogenital distance (measured from the midpoint of the anus to the anterior base of the penis), anoscrotal distance (measured from the midpoint of the anus to the posterior insertion of the scrotum), and detailed examination of the breasts and genitalia.

The relationship between maternal pregnancy urinary phthalate concentration (logarithmically transformed) and anogenital distance was evaluated using general linear models. Analyte concentrations below the limits of detection were considered to be the limit of detection value divided by $\sqrt{2}$. Data were also analyzed using a categorical approach based on 25th and 75th percentiles for age- and weight-adjusted anogenital distance and 25th and 75th percentiles for analyte concentrations. **[Categorical analysis was reported only for the 4 analytes associated with decreased weight-adjusted anogenital distance.]** Potential confounders considered in regression analysis included mother's ethnicity, smoking status, time and season of urine collection, gestational age at time of urine collection, and infant weight at examination. In addition to using individual urinary phthalate monoester concentrations in the analysis, the authors constructed a total phthalate score based on quartiles of individual phthalate concentrations **[using only the concentrations of monobutyl, monobenzyl, monoethyl, and mono-isobutyl phthalate, which had been shown to be associated with weight-adjusted anogenital distance, discussed below]**. Individual phthalate concentrations in the lowest quartile made no contribution to the total phthalate score. One point was given for each quartile above the lowest quartile. The calculation of total phthalate score led to the trichotomizing of samples into categories of low (score 0–1), intermediate (2–10), and high (11–12).

The final regression model, including only age and age^2 as covariates, showed an inverse relationship between logarithmically transformed analyte concentration and weight-adjusted anogenital distance for monobutyl ($P = 0.031$), monobenzyl ($P = 0.097$), monoethyl ($P = 0.017$), and mono-isobutyl phthalate ($P = 0.007$). The regression coefficient for MEHP was –0.051 ($P = 0.833$). The regression coefficient for 5-oxo-MEHP was –0.412 ($P = 0.114$), and the regression coefficient for 5-hydroxy-MEHP was –0.398 ($P = 0.145$). The authors noted that the regression coefficients for the oxidative MEHP metabolites were of similar magnitude to the coefficients for monobutyl and monobenzyl phthalate (–0.592 and –0.390). The authors indicated that DEHP shortens anogenital distance in rodents and that it was not possible to tell if the urinary concentrations of MEHP and its oxidative metabolites failed to be associated significantly with anogenital distance in children because of sample size limitations or because humans and rodents responded differently to DEHP.

Strengths/Weaknesses: The prospective nature of the study and the collection of urine for exposure assessment during pregnancy, reflecting in utero exposure, are strengths as is the measurement of urinary metabolites rather than parent compounds, avoiding contamination issues. The choice of anogenital distance as an endpoint, consistent with a sensitive endpoint in rodents and the masking of clinicians measuring anogenital distance to exposure status are additional strengths. There were, however, few data on the inter-rater reliability of the measurement of anogenital distance or other variables that may be associated with anogenital distance. Methods used to determine independent or combined effects of various phthalates (creation of summary score) were not appropriate for that purpose.

Utility (Adequacy) for CERHR Evaluation Process: This study is very useful for the evaluation process.

Rais-Bahrami et al. *(74)*, sponsored by NIH, examined onset of puberty and sexual maturity parameters in 14–16 year-old adolescents who had been subjected to ECMO as neonates; the

procedure potentially leads to high DEHP exposure. The adolescents included 13 males and 6 females. Measurements taken during physical examinations included height, weight, head circumference, testicular volume, and phallic length. Pubertal staging was conducted according to the Tanner method. Laboratory tests were conducted to assess thyroid, liver, and renal function. Luteinizing hormone (LH) and follicle stimulating hormone (FSH) levels were measured in both sexes, estrogen levels were measured in females, and testosterone levels were measured in males. Except for 1 female with Marfan syndrome, growth percentiles were normal for age and sex. Pubertal development was stated to be normal. **[The authors did not state whether testicular volume and phallic length were normal.]** Laboratory results indicated normal thyroid, liver, and renal function. LH, FSH, testosterone, and estradiol levels were normal for stage of pubertal development. **[A control group of children who did not receive ECMO treatment as neonates was not included for comparison. In addition, the authors did not state the criteria they used for determining if parameters were within normal ranges.]** The study authors concluded that their study ". . . did not show long-term adverse outcome related to physical growth and pubertal development in adolescents previously exposed to DEHP in the neonatal period."

Strengths/Weaknesses: The extensive assessment of endocrine function to supplement Tanner stages is a strength of this study; however, there were no measurements of phthalate exposure, and there was no comparison group to compare to children presumed to be exposed.

Utility (Adequacy) for CERHR Evaluation Process: This study is of minimal utility for the evaluation process.

Colón et al (75), supported in part by the EPA Minority Academic Institutions Traineeship programs, compared blood phthalate levels in premature thelarche patients and controls. Cases consisted of girls between the ages of 6 months and 8 years with premature breast development. Controls consisted of females aged 6 months to 10 years who displayed no evidence of premature sexual development or other endocrine disease. Blood samples from premature thelarche patients were taken between January 1994 and April 1998. **[It is not specified if blood samples were collected from controls during the same time period.]** Forty-one samples were obtained from premature thelarche patients and 35 samples from control patients. **[It was not stated if each sample was obtained from a different subject.]** Levels of phthalates, including DEHP, were measured in serum by GC/MS; numerous blank samples were analyzed to rule out contamination through solvents, water, or medical or laboratory equipment. Phthalates were detected in 28 of 41 samples from premature thelarche patients. DEHP was detected in 25 of the samples at concentrations ranging from 187 to 2098 μg/L (ppb); average concentration was reported at 450 μg/L. MEHP was detected in 5 of the samples at concentrations of 6.3–38 μg/L. In the control group, DEHP was detected in 5 of 35 blood samples at concentrations of 276–719 μg/L; average concentration was reported at 70 μg/L. Di-n-octylphthalate was the only other phthalate detected in 1 control sample. The difference in average blood DEHP level in cases versus controls was found to be statistically significant using the 95% confidence interval. **[Methods of statistical analyses were not discussed.]** Study authors concluded "This study suggests a possible association between plasticizers with known estrogenic and antiandrogenic activity and the cause of premature breast development in a human female population."

[The Expert Panel notes a letter by McKee (76) in response to the Colón et al. study. This letter identified the blood DEHP and MEHP concentrations as being difficult to reconcile with published studies on phthalate blood levels. The very high blood levels of DEHP and low blood levels of MEHP were described as "anomalous" and consistent only with sampling immediately after introduction of substantial amounts of DEHP into the bloodstream, as might occur after a medical procedure. This scenario would not be

consistent with a picture of chronic DEHP exposure levels such as might be hypothesized to affect thelarche. In addition, the possible role of DEHP in premature thelarche was discounted as being inconsistent with the lack of effect of phthalates on timing of female sexual development in experimental animal studies. The Expert Panel agrees with McKee that the DEHP concentrations reported in this study are unreliable.]

Strengths/Weaknesses: This study used a clinically relevant outcome, but phthalates detected in serum specimens may have been unreliable due to laboratory contamination or to medical procedures conducted because of the diagnosis. It was not stated whether phthalate laboratory analyses were conducted blind to case status.

Utility (Adequacy) for CERHR Evaluation Process: This report is not useful because of the lack of confidence in the reported DEHP measurements.

3.2 Experimental Animal Data

3.2.1 Developmental Studies Focusing on Reproductive System and Endocrine Effects

This section examines reproductive or endocrine effects occurring in animals dosed during gestation or during the pre-weaning stage. Studies examining reproductive effects in animals dosed subsequent to the lactational stage ($\geq$ 21 days of age) are summarized in Section 4.

3.2.1.1 In vivo exposures

Gray et al. (77), from the US EPA, examined the effect of perinatal phthalate exposure in rats. It appears that data from this study were also reported in an abstract by Ostby et al. *(78)*. Sprague-Dawley rats were gavage dosed with 0 (corn oil vehicle) or 750 mg/kg bw/day DEHP (99% purity) from GD 14 (GD 1 = day sperm detected) to postnatal day (PND) 3 (PND 1 = postcoital day 23). The experiment was repeated with a second block of animals. In each block of the experiment, there were 7–9 treated dams and 9–10 control dams. Parameters examined in pups (period examined) included body weight (PND 2), anogenital distance (PND 2), testicular histology (PND 2, 9–10, and 13, 3–5 months, and 4–7 months), areolas/nipples (PND 13), preputial separation (beginning on PND 28), mating behavior (adulthood), abnormalities of reproductive organs (3–5 months and 4–7 months), and sperm counts. Statistical analyses were based on litters, and blocks were pooled in cases of identical results. Analyses included one-way analysis of variance (ANOVA) followed by post hoc *t*-tests when statistical significance was obtained. Anogenital distance and organ weight data were covaried with body weight. Categorical data were analyzed by Fisher exact test or chi-squared test.

DEHP treatment resulted in a small reduction in maternal body weight gain. Litter weight at birth was significantly reduced by 15% in the DEHP group, but there was no effect on number of live pups at birth. In DEHP-treated males on PND 2, anogenital distance was significantly decreased by ~30%, with or without adjustment for body weight, and paired testis weights were significantly decreased by 35%. There was no effect on anogenital distance in female pups. Histological examination of testes from DEHP-treated rats on PND 2–3 revealed focal interstitial hemorrhage and multinucleated gonocytes containing 3–5 nuclei or undergoing degenerative changes. Hemorrhagic testes were observed in 7 DEHP-treated males from 3 litters at PND 8–9. Histological examination of testes on PND 9–10 revealed evidence of focal hemorrhage in some testes and extensive coagulative necrosis in other testes of DEHP-treated rats; loss of seminiferous epithelium was observed in areas with hemorrhage or necrosis. Areolas were observed in 87% of DEHP-treated male pups versus none in control pups. DEHP treatment did

not delay the age of preputial separation, but preputial separation was incomplete due to malformations in 19 of 56 treated pups.

DEHP did not appear to affect sexual behavior in adult rats, except that males with malformed penises were unable to achieve intromission. At necropsy, 45 DEHP-treated adult rats from 15 litters were assessed for malformations of reproductive organs, which were observed in 82% of DEHP-treated males. The types of malformations included permanent nipples, clefting of phallus and hypospadias, vaginal pouches, agenesis of prostate, seminal vesicles, or coagulating glands. Sperm production and numbers were said to be unaffected by DEHP treatment **[data not shown]**. Testicular defects included hemorrhage, granuloma, fibrosis, reduced size or atrophy, and non-descent associated with abnormal gubernacula or ligaments. Significant reductions in weight were observed for all male reproductive organs including testis, levator ani plus bulbocavernosus muscle, seminal vesicle, prostate, penis, and epididymis. Liver, pituitary, kidney, and adrenal weights were not affected by DEHP treatment. Serum testosterone levels were unaffected in DEHP-treated rats. The study authors concluded that 750 mg/kg bw/day DEHP severely alters sexual differentiation in an anti-androgenic manner.

Strengths/Weaknesses: This paper reports a high quality comprehensive evaluation of the potential anti-androgenic effects of DEHP in rats when administered at a single dose during a period of critical sexual differentiation, from GD 14 to PND 3. The experiment included a relevant route of exposure, and the authors provided the source and purity of test material. Strengths include good detailed description of methodology, robust numbers of litters evaluated (as high as 16 litters) for the "active" phthalates, appropriate statistical analyses using the litter as the unit of analyses for most endpoints, randomization of animals into blocks, and near complete presentation of data with standard errors where appropriate. Most treatment-related changes were robust and clearly distinguishable from controls, resulting in high confidence for the findings. The authors described a characteristic phthalate phenotype, although they were not the first to do so. The suggestion of a similar mechanism of action for fetal and pubertal male effects based on the structure of active and inactive phthalates was an important contribution. Weaknesses include the single, high dose level, the small sample size for most of the "inactive" phthalate exposures, and the presentation of only fetal incidence for malformation data.

Utility (Adequacy) for CERHR Evaluation Process: The majority of these data are adequate for consideration. The study robustly demonstrates multiple adverse effects on sexual development in males, fetal growth, and maternal toxicity. Because only one dose level was used, a NOAEL was not obtained and a dose-response and benchmark dose evaluation cannot be conducted. The presentation of the pathology findings is troublesome in that no data or comparison to the controls were presented, diminishing the value of this information for the evaluation. However, given the severity of the findings and the support of other endpoints affected, this oversight may be of less importance. The behavioral evaluation is insufficient and cannot be used for risk assessment.

Parks et al. *(79),* from the US EPA, conducted a series of in vivo, ex vivo, and in vitro studies to examine mechanisms of DEHP-induced malformations in rat reproductive organs. A competitive androgen binding study was conducted in monkey cells transfected with the human androgen receptor vector pCMVhAR. Radioactivity was measured following incubation of the cells for 2 hours with 5 nM ^{3}H-R1881 (a synthetic androgen ligand) and DEHP or MEHP at concentrations of 0 or 0.05–10 μM. Neither DEHP nor MEHP competed with R1881 for androgen receptor binding.

In the in vivo study, Sprague-Dawley rats were randomly assigned to groups that were gavage dosed with 0 (corn oil vehicle) or 750 mg/kg bw/day DEHP from GD 14 (GD 1 = day after mating) until necropsy. Rats were killed and necropsied on GD 17, 18, or 20 or PND 2 (PND 1 = day after birth). The study was conducted in 2 blocks, and a total of 4–5 litters per group were examined at each necrospy period. At GD 17, 18, and 20 and PND 2, 1 testis from 2 or 3 males/litter was incubated in media for 3 hours to determine ex vivo testosterone production, and the other testis was used to measure testosterone content. In GD 17, 18, and 20 males, testosterone levels were also measured in the carcasses from which testes were removed (n = 18–20 per group). Testosterone levels were measured by radioimmunoassay (RIA). One testis from each of 4 DEHP-treated and 6 control PND 2 males was fixed in 5% glutaraldehyde for histopathological examination. One testis from each of 4 control and 5 DEHP-exposed PND 20 males and an unspecified number of DEHP-exposed PND 3 males from a parallel study was stained for 3β-hydroxysteroid dehydrogenase, which is specific for Leydig cells. Anogenital distance was measured in all male and female offspring on PND 2. Litter means were used in statistical analyses. Data were analyzed by ANOVA followed by 2-tailed t-tests if ANOVA resulted in significant findings. Testicular histopathological findings were analyzed by Fisher exact test.

Maternal weight gain during gestation was significantly reduced in the DEHP-treated group. Number of live pups at birth was not significantly affected by DEHP treatment. Ex vivo testicular testosterone production in GD 17, 18, and 20 and PND 2 offspring from DEHP-exposed groups was significantly lower compared to control groups. Testicular testosterone content in DEHP-exposed offspring and pups was reduced by 60–85% compared to controls examined at each necropsy period; the effect was statistically significant at all time points except GD 20. **[It appears a footnote regarding GD 20 is missing in Table 1 of the study.]** Whole body testosterone levels were significantly lower in DEHP-exposed fetuses on GD 17 (71% lower than controls) and 18 (47% lower than controls), but the reduction on GD 20 was not significant. Significant reductions in testis weight were noted in the DEHP group on GD 20 (18% lower than controls) and PND 2 (49% lower than controls). Body weights of DEHP-exposed pups were described as 23% lower than controls on PND 2, but statistical significance was not achieved. Testis weights adjusted for body weights were significantly decreased in PND 2 pups exposed to DEHP. Anogenital distance was significantly reduced by 36% in PND 2 males compared to controls but was not affected in female pups exposed to DEHP. Histopathological examination of PND 2 testes of DEHP-treated rats revealed an increased number of enlarged and multinucleated gonocytes and aggregates of hyperplastic Leydig cells. 3β-Hydroxysteroid dehydrogenase staining confirmed the presence of Leydig cell aggregates in DEHP-exposed males on GD 20 and PND 2. In contrast, 3β-hydroxysteroid dehydrogenase staining revealed an even dispersion of Leydig cells and less intense staining in testes of control fetuses and pups.

The study authors concluded that treatment with 750 mg/kg bw/day DEHP inhibited testosterone production in male pups during the period of sexual differentiation, and this inhibition was a likely cause of malformations observed in other studies. Malformations likely result from a mechanism that does not directly involve the androgen receptor.

Strengths/Weaknesses: This report includes a good, detailed description of the methods, appropriate route of exposure, and statistical methods using the litter as the unit of analyses. The authors provided excellent use of controls in the evaluation of pathology. An appropriate exposure period was used for male effects. This study demonstrated clear effects on fetal whole body and fetal and postnatal testicular testosterone levels in male offspring, testes weight, anogenital distance, and histopathology of the testes following maternal gestational or gestational and postnatal exposure, and provided clear evidence that DEHP and its principal metabolite do

not bind to the androgen receptor. The description of the time course for fetal testosterone alterations is an additional strength. Weaknesses include the single, high dose level and the small sample size. The samples size was sufficient to demonstrate robust effects of treatment but not to provide high confidence in lack of effect. There was no purity information on the test material.

Utility (Adequacy) for CERHR Evaluation Process: This study is adequate in providing mechanistic information for use in the evaluation process. Because only one dose level was used, the study cannot establish a NOAEL and is not suitable for dose-response or benchmark dose evaluation. Given the small sample size, there is low confidence in negative findings.

Akingbemi et al. *(80)*, supported by NIEHS, evaluated the effect of DEHP on Leydig cell function in male Long-Evans rats exposed in utero, during nursing, or during post-weaning stages (the post-weaning results are presented in Section 4.2.2). Pregnant rats were exposed to DEHP (>99% purity) on GD 12–21 **[plug day not indicated]**, and lactating rats were exposed on PND 1–21 (day after birth = PND 1). DEHP was administered to dams by gavage in corn oil at 0 or 100 mg/kg bw/day. Males were obtained for evaluation on PND 21, 35, or 90 (n = 7 dams/group/stage) **[no information was provided on culling or rearing]**. Male offspring were decapitated within 24 hours of the final dose, and trunk blood was collected for measurement of LH and testosterone by RIA. Testes and seminal vesicles were weighed, and testicular interstitial fluid was collected for measurement of fluid testosterone by RIA. Testicular histology was evaluated. Cultures of Leydig cells or, in 21-day-old animals, progenitor Leydig cells, were prepared from testes by Percoll density gradient preceded in 90-day-old rats by centrifugal elutriation. The resulting preparations were 90% pure for progenitor Leydig cells and 95–97% pure for PND 35 or 90 Leydig cells, as evaluated by staining for 17-hydroxysteroid dehydrogenase. Cultures were evaluated after 3-hour incubation with and without a maximally stimulating concentration of ovine LH. Testosterone was measured in the medium. **[According to the methods section, the activity of different enzymes in the testosterone biosynthesis pathway were evaluated by incubating Leydig cells for 3 hours with saturating concentrations of substrate for the enzyme of interest; however, results of these experiments were given only for males treated during the post-weaning period (summarized in Section 4.2.2).]** Statistical analysis was by ANOVA and Duncan multiple range test.

There were no effects of treatment during gestation or lactation on dam weight or weight gain or on offspring weight. Offspring testis and seminal vesicle weights were also not affected by treatment during either developmental period. Serum testosterone was reduced 31–33% and serum LH was reduced 50–64% **[estimated from a graph]** in 21- and 35-day-old males exposed to DEHP during gestation. There were no prenatal DEHP effects on serum testosterone or LH in 90-day-old males. Prenatal exposure to DEHP resulted in decreased testosterone production by cultured progenitor Leydig cells obtained from 21-day-old males. Basal testosterone production was reduced 47%, and LH-stimulated testosterone production was reduced 56%. There were no treatment effects on cultured Leydig cells derived from 35- and 90-day-old offspring. Lactational exposure to DEHP was associated with a 13% decrease in serum testosterone on PND 21. There were no significant changes in serum LH on PND 21 or in testosterone or LH on PND 35 or 90. **[No results were presented for cultured Leydig cells derived from males exposed during lactation.]** Testicular histology was described as normal in all treatment groups.

The authors concluded that exposure to DEHP during gestation or lactation resulted in suppression of pituitary LH in the presence of reduced serum testosterone, and that growing rats were more susceptible to the effects of developmental exposure than adult rats.

Strengths/Weaknesses: This report contains good descriptions of experimental design and methods with some exceptions. The studies used an appropriate route and time of exposure and chemical source and purity were described. Multiple dose levels in the second study (discussed in Section 4.2.2) allowed for dose-response analyses. The comparison of responses to postnatal exposure at 3 different ages is a strength. Weaknesses include inadequate detail on the numbers of animals and numbers of litters per group used for histopathologic examination. The litter was not utilized as the unit of analyses following maternal/gestational exposure. For prepubertal and young adult rats, animals were randomly selected and assigned to treatment groups, but correction for potential litter effects was not conducted. Although the lack of control for litter effects is a weakness in this study, it is less compromising for prepubertal rats directly dosed with DEHP, because litter effects diminish somewhat with age post-weaning, and the animals were given a standardized dose based on individual body weight. The single, high dose level used in the first experiment is a weakness.

Utility (Adequacy) for CERHR Evaluation Process: The histopathology results are of limited value because no data were presented, and it was not clear how many animals from each treatment group were evaluated. The gestational and lactational results from this study are not useful for the evaluation because the study design did not control for litter effect following maternal exposure. The lactational data were not presented. The ex vivo testosterone production data are not useful for the evaluation process due to the artificial in vitro environment in which these data were generated, which has an uncertain application to human risk. The enzyme activity and testosterone production information are useful for providing insight into potential mechanisms of action (Section 4.2.2).

Moore et al. *(81)*, supported by NIH and University of Wisconsin, examined rat sexual development in offspring of dams dosed with DEHP during gestation and lactation. In an experiment conducted in 2 blocks, at least 8 pregnant Sprague-Dawley rats/group were orally dosed **[presumed gavage]** with DEHP (99% purity) at 0 (corn oil vehicle), 375, 750, or 1500 mg/kg bw/day from GD 3 (GD 1 = day after sperm detected) to PND 21. One group of rats was dosed with 3000 mg/kg bw/day in the first block of the study, but that dose was not used in the second block due to excess toxicity consisting of nearly complete prenatal or postnatal mortality. Dams were allowed to litter, and the litters were adjusted to 10 pups at 1–2 days following birth. Litters were maintained at 10 pups by replacing any pups that died with pups from litters exposed to the same or lower concentrations of DEHP; data from replacement pups were not reported. Parameters examined in all pups (time period examined) included pup weight (PND 1, PND 7, and then weekly), anogenital distance (PND 1), areolas (from PND 11), vaginal opening (from PND 24), time to first estrus (starting from vaginal opening), preputial separation (from PND 38), and male sex organ weight (PND 21, 63, and 105). In PND 63 rats, 1 epididymis and testis were fixed in neutral-buffered formalin, and the other testis and epididymis were used to determine daily sperm production. Sexual behavior with a sexually receptive female rat was assessed in males that were later necropsied on PND 105. The litter was considered the experimental unit in statistical analyses that included Levene test for homogeneity of variance, ANOVA, least significant difference test, chi-squared test, and/or Fisher exact test.

Results achieving statistical significance or displaying dose-response relationships are summarized in Table 19. DEHP treatment reduced prenatal maternal weight gain at the middle and high dose. There was no significant effect on implantation sites, though the number appeared to be slightly reduced by DEHP treatment. All the rats with implantation sites gave birth to litters except for 1 mid-dose and 2 high-dose rats. Number of pups born was reduced at the high dose, and postnatal survival was decreased at the middle and high dose. Adult male offspring exposed to DEHP experienced a 6% reduction in body weight at the middle dose and 12% reduction at the

high dose **[data not shown]**. An 8% reduction in body weight of adult female offspring of the high-dose group was reported as not significant. **[Data were not shown. A significant reduction in female body weight was reported for day of vaginal opening, as discussed below.]**

DEHP treatment caused numerous effects on the reproductive systems of male rats, outlined in detail in Table 19. Areolas or nipples were not observed in any control male rats but were increased according to dose in all treated rats. Effects first noted in male rats of the mid-dose group were reduced anogenital distance, increased numbers of undescended testes, reduced sperm counts, and agenesis of anterior prostate. Incidence of incomplete preputial separation was significant at the high dose, but the authors considered the effect to be biologically significant at all doses due to the rarity of the effect in rats. Agenesis of prostate, seminal vesicles, and epididymis was noted in some treated rats. Agenesis of anterior prostate was significant at the middle and high dose; the study authors suggested that the effect was biologically significant at the low dose due to rarity of prostate agenesis. DEHP treatment reduced absolute weights of testes, epididymides, and glans penis at PND 21, 63, and/or 105, and in most cases, statistical significance was obtained at the middle and high dose. Absolute weight effects are outlined in Table 19. In most cases, effects on relative weights were similar in terms of direction and statistical significance of effect. Weights of accessory male organs, which are not illustrated in Table 19, were also reduced by DEHP treatment. The organs affected (day and dose that statistical significance was achieved for absolute organ weight) were ventral prostate (PND 21: middle and high dose; PND 105: mid dose), dorsolateral prostate (PND 21 and 105: middle and high dose; PND 63: high dose), anterior prostate (PND 21: all doses; PND 63 and 105: middle and high dose), and seminal vesicles (PND 21: middle and high dose). Effects on relative organ weights were similar in most cases. In sexual behavior tests, there were fewer rats from all dose groups that did not mount, intromit, and/or ejaculate. The authors stated that statistical significance was not obtained due to the small numbers of animals tested (n = 7–8 at control and 2 lower dose levels and n = 2 at high dose level).

Statistically significant and dose-related effects for female offspring are also listed in. In female pups, DEHP treatment had no effect on anogenital distance. At the high dose, vaginal opening was described as having occurred slightly earlier than in control rats, but age at first estrous was described as slightly higher; neither effect was statistically significant. Body weight of high-dose females was 68% that of control body weight on the day of vaginal opening, and the effect was statistically significant. The study authors attributed the effect to DEHP-induced toxicity and not to an estrogenic effect.

[CERHR estimated benchmark doses for endpoints when there was evidence of a dose-response relationship and for which the authors reported sufficient data for benchmark dose modeling. Benchmark dose values are presented in Table 20.[1]] The study authors identified a LOAEL of 375 mg/kg bw/day for this study based on a significant decrease in anterior prostate weight and an increase in permanent nipple retention. Other biologically significant effects observed by study authors at 375 mg/kg bw/day were non-descent of testes, incomplete preputial separation, and agenesis of accessory sex organs. The study authors noted that DEHP exposure adversely affected reproductive system development and sexual behavior in male rats, but there was no evidence of estrogenic activity in female rats.

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[1]Benchmark doses are used commonly in a regulatory setting; however, they are used in this report when the underlying data permit their calculation, and are only supplied to provide 1 kind of description of the dose-response relationship in the underlying study. Calculation of a benchmark dose in this report does not mean that regulation based on the underlying data is recommended, or even that the underlying data are suitable for regulatory decision-making.

Strengths/Weaknesses: This paper includes a good, detailed description of methodology and statistical analyses using the litter as the unit of analyses. The study was well-designed, using an appropriate route and timing of exposure and multiple dose levels. Even thought the sample size was small, there was sufficient magnitude of effects to obtain statistical significance. The study demonstrated a pattern of effects consistent with other study findings and the establishment of NOAELs/LOAELs. The dose-response data are appropriate for benchmark dose evaluation. It is a strength that animals were followed into early adulthood. The observation of effects on reproductive behaviors in the absence of gross external changes suggested additional effects on the central nervous system. This study reinforced the increased sensitivity of the fetal male compared to the pubertal or adult male, although only by reference to existing literature.

Weaknesses include the small sample size, and negative effects cannot be accepted with high confidence. For example, there appeared to be a significant biological effect of reduced implantation sites at 1500 mg/kg, but no statistical significance. Professional experience and judgment would lead to the conclusion of an effect on the number of implantation sites. The use of relatively high dose levels is another weakness. The authors used a post hoc statistical design for grouping of all reproductive abnormalities/defects and for behavioral effects. Post hoc (after generation and visual assessment of the data) statistical analyses are generally considered inappropriate, however, because the signal for reproductive abnormalities/defects is overwhelming (60–80% of litters affected in the DEHP group compared to zero in the control), this oversight has no significance for these endpoints. It is a common practice when conducting embryo/fetal toxicity studies to group malformations and variations by system or type (external, visceral, or skeletal; organ system). For the behavioral assessment, the post hoc grouping of findings across DEHP treatment groups was inappropriate and not valid for risk assessment, but valuable for hypothesis generation.

Utility (Adequacy) for CERHR Evaluation Process: This study was well conducted and used a comprehensive battery of relevant developmental endpoints, multiple dose levels, and a relevant route of exposure during critical periods of sexual development. Even though sample size and power were limited, clear treatment-related (and in many cases dose-responsive) effects on maternal toxicity, reproductive parameters, measures of sexual differentiation/development, and sexual function were identified. Given the variability in response, small sample size, and post hoc grouping of results across treatments, the behavioral data are not appropriate for the evaluation process. The negative findings are not viewed with a high confidence level given the small sample size.

Table 19. Results Achieving Statistical Significance or Dose-response Relationships Following DEHP Prenatal and Lactational DEHP Exposure

Endpoint	Maternal DEHP dose (mg/kg bw/day)			
	0	375	750	1500
Maternal prenatal weight gain, g (% of control value)	128 ± 4	123 ± 7 (96)	99 ± 10* (77)	87 ± 13* (68)
Parturition incidence (%)	100	100	89	75
Pups born/dam (% of control value)	12.5 ± 1.0	11.4 ± 0.8 (91)	9.6 ± 1.3 (77)	7.7 ± 1.4* (62)
Pups surviving/dam (% of control value)	10.9 ± 1.0	9.8 ± 0.8 (90)	7.5 ± 1.3* (69)	5.0 ± 1.3* (46)
Mean male anogenital distance, mm[a]	3.5	3.3	3*	2.5*
Mean no. areolas per male[a]	0	2	7*	9.5*
Mean % litters containing males with:				
areolas or nipples on PND 14[a]	0	62*	100*	100*
areolas or nipples as adults[a]	0	50*	85*	100*
incomplete preputial separation[a]	0	10	25	80*
undescended testis on PND 21[a]	0	42	75*	100*
with undescended testis as adults	0	25	58	40
Daily sperm production, 10^6/testes (% of control value)	34.2 ± 1.5	36.5 ± 1.2 (107)	25.6 ± 4.5 (75)	24.4 ± 5.4 (71)
Epididymal sperm number in 10^6/cauda (% of control value)	55.5 ± 3.7	46.5 ± 5.1 (84)	29.8 ± 8.7* (54)	19.3 ± 7.5* (35)
Litters (pup) with abnormality/no. examined				
ventral prostate agenesis	0/8 (0/42)	1/8 (1/32)	2/8 (5/29)	2/5 (3/12)
dorsolateral prostate agenesis	0/8 (0/42)	1/8 (1/32)	0/8 (0/29)	1/5 (2/12)
anterior prostate agenesis	0/8 (0/42)	1/8 (1/32)	5/8* (9/29)	4/5* (6/12)
seminal vesicle agenesis	0/8 (0/42)	0/8 (0/32)	0/8 (0/29)	2/5 (2/12)
Litters with reproductive defects, %[a,b]	0	65*	88*	100*
Index of abnormalities, %[a,c]	0	18*	55*	75*
Absolute testes weight, % of control value				
PND 21		90	78*	62*
PND 63		103	73*	59*
PND 105		99	71	39
Absolute epididymides weight, % of control value				
PND 63		98	66*	69*
PND 105		91	61*	55*
Absolute glans penis weight, % of control value				
PND 21		90	82*	71*
PND 63		97	89*	83*
PND 105		97	86*	79*
Age at vaginal opening, days	31.1 ± 0.9	29.7 ± 0.9	29.7 ± 1.2	27.2 ± 1.1
Body weight at vaginal opening, g (% of control value)	94 ± 6	87 ± 6 (92)	86 ± 9 (91)	64 ± 9* (68)
Age at first estrus in days	33.5 ± 0.3	33.1 ± 0.4	35.8 ± 2.0	34.4 ± 0.7
Body weight at first estrus, g (% of control value)	108 ± 4	105 ± 2 (97)	116 ± 11 (107)	98 ± 2 (91)

From: Moore et al. *(81)*. Mean ± SEM unless otherwise stated.
*$P < 0.05$.
[a]Estimated from graph by CERHR; [b]reproductive defects included missing, malformed, or small sex organs; incomplete preputial separation; or undescended testis; [c]index of abnormalities is score based on missing, pathological, or small reproductive organs; presence of nipples or undescended testis; incomplete preputial separation; and failed ejaculation.

Table 20. Benchmark Dose Values for Offspring of Rats Exposed to DEHP During Gestation and Lactation

Endpoint		Benchmark dose (mg/kg bw/day)[a]			
		BMD_{10}	$BMDL_{10}$	$BMD_{1\,SD}$	$BMDL_{1\,SD}$
Maternal prenatal weight gain		433	317	754	509
Pups born/dam		378	269	872	565
Pups surviving/dam		269	205	696	479
Absolute testis weight	PND 21	384	298	562	400
	PND 63	421	283	452	258
	PND 105	374	204	342	203
Epididymis weight	PND 63	394	291	395	233
	PND 105	262	196	220	135
Glans penis weight	PND 21	503	393	526	379
	PND 63	806	623	445	321
	PND 105	642	500	278	171
Daily sperm production/testes		490	289	686	389
Epididymal sperm number		213	167	612	428
Body weight at vaginal opening		780	343	1157	611

From: Moore et al. *(81)*.

[a]The BMD_{10} is the benchmark dose associated with a 10% effect, estimated from a curve fit to the experimental data. The $BMDL_{10}$ represents the dose associated with the lower 95% confidence interval around this estimate. A 10% alteration in a continuously distributed parameter is an arbitrary benchmark that may not be comparable to a similar alteration in any other endpoint. The $BMD_{1\,SD}$, which represents an alteration equivalent to 1 SD of the control distribution, may permit more appropriate comparisons of the responses of continuously-distributed parameters. Benchmark doses are used commonly in a regulatory setting; however, they are used in this report when the underlying data permit their calculation, and are only supplied to provide 1 kind of description of the dose-response relationship in the underlying study. Calculation of a benchmark dose in this report does not mean that regulation based on the underlying data is recommended, or even that the underlying data are suitable for regulatory decision-making. Values were calculated using the power model by CERHR using EPA Benchmark Dose Software version 1.3.2. The program offers models based on homogeneity of variance and CERHR was guided by the program in this regard.

Borch et al. *(82)*, support not indicated, conducted a series of studies in Wistar rats to examine anti-androgenic effects of DEHP (99% purity) alone or in combination with diisononyl phthalate or diethylhexyl adipate. Diisononyl phthalate was examined alone in some cases, but this summary focuses on DEHP. Dams were gavage dosed with vehicle (peanut oil) or DEHP in peanut oil during gestation and/or lactation. Endpoints examined in male offspring included hormone levels in testes or blood and ex vivo testicular testosterone production. To measure ex vivo testosterone production, the left testis was incubated in media for 3 hours and the supernatant was saved for testosterone analysis. Following extraction from incubation media, testis, or blood, quantification of hormone levels was performed using fluorometric or immunofluorometic methods. Testicular testosterone production was measured in testes from 2 males per litter, and testicular testosterone content was measured in testes from 1 male per litter. Plasma samples were pooled from 1 or 2 litters in the case of fetal or immature offspring or were obtained from 9–16 males per group in the case of mature offspring. Data were evaluated by ANOVA, analysis of covariance (ANCOVA), Dunnett test, and /or Pearson correlation. Litter was included as an independent random factor in ANOVA analyses.

The first study appears to have been previously reported in an abstract *(83)*. Approximately 8 dams/group were gavage dosed during gestation with vehicle, 300 mg/kg bw/day DEHP, or 300 mg/kg bw/day DEHP + 750 mg/kg bw/day diisononyl phthalate on GD 7–21. **[Criteria for determining day of gestation were not stated but it is assumed that GD 1 was the day following mating, as in study 2 described below.]** Endpoints examined in GD 21 male fetuses included testicular testosterone content and production and plasma testosterone and LH levels. Compared to control values, testicular testosterone content and production were significantly reduced in the DEHP and DEHP + diisononyl phthalate groups. Plasma testosterone was significantly reduced, and plasma LH was significantly increased in the DEHP + diisononyl phthalate group; similar effects on plasma testosterone and LH levels were described in groups receiving DEHP or diisononyl phthalate alone, but statistical significance was not achieved. Factorial statistical analyses revealed no significant interactions between DEHP and diisononyl phthalate.

In the second study, 16 pregnant Wistar rats per group were gavage dosed with vehicle, 750 mg/kg bw/day DEHP, or 750 mg/kg bw/day DEHP + 400 mg/kg bw/day diethylhexyl adipate from GD 7 to PND 17. GD 1 was the day following mating, and PND 1 was the day following birth. Eight dams per group were randomly selected and killed on GD 21. The fetuses were also killed for collection of blood and testes. The remaining 8 dams per group were allowed to litter; anogenital distance was measured in their male offspring on PND 3, and nipple retention was assessed on PND 13. In GD-21 male fetuses exposed to DEHP or DEHP + diethylhexyl adipate, testicular testosterone content and production and plasma testosterone levels were significantly reduced compared to the control group. Plasma LH level was significantly increased in GD 21 fetuses exposed to DEHP. Plasma LH levels were not measured in the DEHP + diethylhexyl adipate group. Anogenital distance was significantly reduced in PND 3 male offspring from the DEHP and DEHP + diethylhexyl adipate groups, and the effect was not related to birth weight. Numbers of nipples per animal were significantly increased in PND offspring from the DEHP group, but the increase in the DEHP + diethylhexyl adipate group was not statistically significant.

In the third study, 80 Wistar rats were randomly assigned to 1 of 4 groups that were treated with DEHP 0, 300, or 750/kg bw/day or DEHP 750 mg/kg bw/day + diethylhexyl adipate 400 mg/kg bw/day from GD 7 to PND 17. Blood and testes were collected from male offspring killed on PND 22 and PND 190. In PND 22 offspring treated with 750 mg/kg bw/day DEHP, there was a dose-related, significant reduction in serum inhibin B level. A non-significant "tendency" for increased serum FSH levels in the DEHP-treated groups was described by the study authors. There were no significant findings for serum LH levels, but about half the litters in the 750 mg/kg bw/day DEHP-treated group had LH levels several-fold higher than those of control rats. Correlations were noted between serum LH and serum FSH ($r = 0.61$, $P < 0.001$) and serum FSH and serum inhibin B ($r = -0.51$, $P < 0.001$). There were no significant differences in plasma testosterone levels on PND 22. No statistically significant differences in serum testosterone levels, testicular testosterone content, or serum inhibin B levels were observed in PND 190 rats.

The study authors concluded that in neonatal rats, endocrine-modulated effects following gestational or lactational exposure to DEHP were similar to those previously reported in the literature, including a reduction in anogenital distance and an increase in nipples. However, hormonal effects were less evident in prepubertal and adult animals. Administration of diisononyl phthalate or diethylhexyl adipate in combination with DEHP caused no significant modulation of endocrine effects.

Strengths/Weaknesses: The use of the Wistar rat was a strength in extending the evaluation of DEHP to a strain different from that used in most other studies. The paper included a good

detailed description of methods including test material source and purity, appropriate statistical evaluation of the data using the litter as the unit of analyses, and nesting of individual animals/litter. Study 2 demonstrated clear treatment-related effects on plasma testosterone and LH in fetuses following maternal gestational exposure to DEHP 750 mg/kg bw. Although the sample size was small (n = 2–6 samples/group), the magnitude of the DEHP effect was sufficient to show statistical significance. Gestational DEHP exposure to 750 mg/kg bw also resulted in decreased anogenital distance and increased number of nipples per male. Study 3 employed multiple dose groups (300 and 750 mg/kg DEHP), allowing for a dose-response evaluation. Maternal animals were dosed from GD 7 through PND 17, the period of sensitivity for male sexual development. The only treatment-related effect noted following maternal exposure from GD 7 through PND 17 was decreased inhibin B on PND 22, demonstrating a Sertoli cell effect. The small sample size (< 8 litters/group/time point) was a weakness in these studies. Ex vivo testosterone production is of questionable relevance to human risk, especially when no in vivo plasma testosterone or LH changes were noted. Study 1 demonstrated no treatment-related effects on plasma testosterone and plasma LH in 21-day-old rat fetuses following maternal gestational DEHP exposure at 300 mg/kg bw. However, given the small sample size (n = 3–7), there is a low level of confidence in the lack of findings. In studies 1 and 2, a single dose level of 300 or 750 mg DEHP/kg bw was used, precluding a dose-response evaluation.

Utility (Adequacy) for CERHR Evaluation Process: Studies 1 and 2 utilized single doses of DEHP and have low value for a quantitative evalution. The results from these studies support findings reported by other authors. Study 3 utilized multiple doses of DEHP (300 and 750 mg/kg bw/day) during a critical period of male sexual development and is useful in the evaluation process. The ex vivo testosterone production data are of questionable relevance to human risk assessment, having no direct human correlate. This assay is best used for studying potential mechanisms/modes of action and for screening for potential activity.

Jarfelt et al. *(84)*, supported by the Denmark Directorate for Food, Fisheries and Agro Business, evaluated the effects of perinatal exposure of Wistar rats to DEHP with or without diethylhexyl adipate. Timed-mated pregnant animals were treated by gavage from GD 7 to PND 17 **[plug day unspecified; date of birth = PND 0]**. Dose groups were vehicle control, DEHP 300 mg/kg bw/day, DEHP 750 mg/kg bw/day, and DEHP 750 mg/kg bw/day + diethylhexyl adipate 400 mg/kg bw/day (n = 20/group). Chemical purity was 99%. Unadjusted litters were raised by their dams until weaning on PND 21, after which 1 male and 1 female per litter were retained. Anogenital distance was assessed on PND 3, and retention of nipples/areolae was assessed on PND 13. Retained offspring were observed for vaginal opening and balano-preputial separation, and males underwent evaluation of epididymal sperm parameters and testicular histopathology on PND 190. **[Later in the Methods section, histopathologic evaluation was described for 14–16 adult males representing 14–16 litters.]** Non-retained pups and dams were killed on PND 22 and evaluated for macroscopic lesions, and 3–5 males/litter underwent histopathologic evaluation of the testes. **[Later in the Methods section, histopathologic evaluation of testes was described for about 5% of PND 22 males, representing 10 litters. The results section presents histolopathology data for 18–21 PND 22 males/dose group.]** Testes were fixed in Bouin fluid (half of PND 22 testes and all adult testes) or in formalin (half of PND 22 testes). Embedding material was not specified for testes. Staining was hematoxylin and eosin. Immunohistochemistry for 3β-hydroxysteroid dehydrogenase and smooth muscle actin was performed using 1 section/testis. Brain, liver, kidney, adrenal, testis, epididymis, seminal vesicle, ventral prostate, bulbourethral gland, and levator ani/bulbocavernosus muscle weights were recorded in all males. Histologic sections of accessory sex organs were prepared for 10 males representing 10 litters. Statistical analysis was performed using ANOVA with post hoc Dunnett test or, for data not satisfying conditions for ANOVA, Kruskall-Wallis or Fisher test. Litter was

included in the analysis. **[Data were shown in the results for 11–15 litters/dose group, with 12 litters in the control group, although 20 timed-mated animals/dose group had been treated. In the group receiving DEHP + diethylhexyl adipate, there were 3 dams with total litter loss. The other missing litters were not explained.]**

Results are summarized in Table 21. Although there were no significant alterations in sperm count and motility parameters, the study authors reported that "a few animals" were severely affected with regard to these parameters. The study authors indicated that males exposed to DEHP with or without diethylhexyl adipate had histologically normal testicular tissue with small foci of malformed tubules associated with interstitial cell hyperplasia. Within these malformed tubules, the seminiferous epithelium was disorganized with decreased spermatogenesis and tubular anastomoses. Immunohistochemistry for 3β-hydroxysteroid dehydrogenase and smooth muscle actin showed Leydig cells inside the malformed tubules. The authors called attention to the higher incidence of malformed tubules among young animals, and they concluded that "dysgenic" testicular tissue was surrounded by normal tissue during pubertal testis growth. There were no abnormal histopathologic findings in accessory sex organs in PND 22 males and only 4 males with mild prostate changes among the 3 DEHP-exposed dose groups on PND 190.

The authors concluded that their study confirmed the anti-androgenic effects of DEHP identified in previous studies, and they called attention to the variability in response among their animals.

Table 21. Outcomes after Perinatal Exposure of Rats to DEHP With or Without Diethylhexyl Adipate

| Endpoint | Treatment group (mg/kg bw/day to the dam) | | | DEHP benchmark dose[a] (mg/kg bw/day to the dam) | | | |
	DEHP 300	DEHP 750	DEHP 750 + DEHA 400	BMD_{10}	$BMDL_{10}$	$BMD_{1\,SD}$	$BMDL_{1\,SD}$
Maternal pregnancy weight gain	↔	↔	↓21%				
Birth weight, males	↔	↓8%	↓9%	770	701	735	405
females	↔	↓8%	↓11%	758	638	750	533
Litter size	↔	↔	↓28%				
Postnatal death	"↑" 4-fold	"↑" 5-fold	↑17-fold	622	127	754	504
Postimplantation loss	↔	↑2.3-fold	↑3-fold	72	13	1127	574
Male anogenital distance	↓14%	↓17%	↓17%	432	315	338	179
Male retained nipples	↑39-fold	↑52-fold	↑39-fold	BMD computation failed			
Sperm/g cauda	↔	↔	↔				
Sperm motility parameters	↔	↔	↔				
Macroscopic malformations	↔	↔	↔				
PND 22 body weight	↔	↔	↔				
paired testis weight	↔	↓8%	↔	748	538	761	652
other organ weights	↔	↔	↔				
abnormal testis histology	↑2.6-fold	↑2.6-fold	↔	126[b]	83		
PND 190 body weight	↔	↔	↔				
ventral prostate weight	↓20%	↓21%	↓17%	388	250	753	454
sexual muscle weight	↓15%	↓18%	↓16%	428	290	635	406
other organ weights	↔	↔	↔				
abnormal testis histology[c]	44%	27%	31%	359[b]	210		

DEHA = diethylhexyl adipate. ↑, ↓, ↔ Statistical increase, decrease, no change compared to control. "↑" Increase identified by study authors, although not statistically different from control.
[a]Benchmark dose calculations performed using the groups treated with control and DEHP only. See footnote to Table 20 for information on the benchmark dose calculations; [b]logistic model; [c]control rate 0%.

Strengths/Weaknesses: Multiple doses of DEHP were used (300 and 750 mg/kg), allowing for dose-response analyses. This study provided a moderately comprehensive evaluation of reproductive and developmental effects following maternal gestational and lactational exposure during the critical period of male sexual development. Assessment of offspring was carried out through sexual maturity. The paper presented good detail on general methodology and test material source and purity, with the exception of suboptimal tracking of litters and lack of consistent use of the litter as the unit of analyses. When multiple pups/litter were used, the litter was included as an independent random and nested factor; however, it was not clear in the paper when, and for what specific parameters, litter was used as an analysis factor. Clear treatment-related findings in male offspring (anogenital distance, nipple retention, reproductive organ weights) were apparent and adequately analyzed. These results also supported findings from other investigators. The use of the Wistar rat was a strength in extending the evaluation of DEHP to a strain different from that used in most other studies. Weaknesses include the relatively high DEHP dose levels that were used. Because the pathology data apparently were not analyzed on a litter basis, these data are not optimal for benchmark dose evaluation. There is significant confusion as to the sample size used for specific parameters and the lack of litter as the unit of analyses for the pathologic data. The authors stated that 1 male and 1 female/litter were kept after weaning (PND 21) to investigate sexual maturation, sperm quality, and histopathology of the testes at adulthood, but this statement contradicts a subsequent statement that "sixteen animals per group were used, one to two males per litter," in reference to sperm count evaluations and the statement that 1–4 males per litter from 10–16 litters were used to analyze sperm quality at PND 190 and terminal body weight. Table 1 of the paper lists only 11–15 litters/group, yet the methods describe males representing 14–16 litters. With the exception of addressing 3 litters with total loss of pups, there is no mention of the loss of litters in the remaining dams. A total of 27 litters are unaccounted for, assuming the 3 litters with total pup loss are included in Table 1 of the paper.

Utility (Adequacy) for CERHR Evaluation Process: The dose-response data for reproductive results (postnatal death, postimplantation loss), offspring growth, nipple development, anogenital distance, sperm quality, and organ weights are suitable for use in the evaluation process. Although NOAELs were not observed for some of these endpoints, the benchmark dose methodology can be applied. The pathology data cannot be used for the evaluation because the litter effect was not controlled and findings in the DEHP groups did not demonstrate a dose-response (likely due to litter effect). The pathology data should be considered supportive information.

Borch et al. *(85)*, supported by the Denmark Directorate for Food, Fisheries and Agro Business, evaluated early testicular effects of perinatal exposure to DEHP with or without diethylhexyl adipate in Wistar rats. In the first experiment, pregnant females were treated by gavage with vehicle, DEHP 750 mg/kg bw/day or DEHP 750 mg/kg bw/day + diethylhexyl adipate 400 mg/kg bw/day beginning on GD 7 (plug = GD 0; n = 18/dose group). Chemicals were of 99% purity. On GD 21, 8 dams/group were killed and fetal testes were harvested. The remaining 8 dams/group continued to receive treatment until PND 17. These animals were permitted to litter. Male offspring were killed on PND 26 (birth = PND 0), and testes were harvested.

A second experiment used 20 pregnant animals in each of 4 dose groups: vehicle control, DEHP 300 mg/kg bw/day, DEHP 750 mg/kg bw/day, and DEHP 750 mg/kg bw/day + diethylhexyl adipate 400 mg/kg bw/day. Treatment was from GD 7 through PND 17. On PND 22, 3 males/litter were killed and testes were harvested. On PND 190, 1 or 2 males/litter were killed and testes harvested.

Of the testes collected on GD 21, 14–19/dose group (2–4/litter) were fixed in formalin, embedded in paraffin, and sections were stained with hematoxylin and eosin for light microscopy. Ten testes/dose group (1 or 2/litter) from PND 22 and PND 26 animals were processed in the same manner. Another 10 testes from these age groups as well as 16 testes/dose group (1 or 2/litter) were fixed in Bouin solution, and stained with hematoxylin and eosin for light microscopy **[embedding material not specified]**. Tubule diameters were measured, and a 10% increase over the control maximum was defined as enlarged. Terminal deoxynucleotidal transferase-mediated dUTP nick-end labeling (TUNEL) staining was performed using a commercial kit and immunostaining was performed for caspase-3, proliferating cell nuclear antigen (PCNA), histone H3, anti-Müllerian hormone, 3β-hydroxysteroid dehydrogenase, vimentin, and smooth muscle actin. Caspase-3 activity was measured in 10 testes/dose group (from 5–10 litters/group) at GD 21, PND 22, and PND 26. **[The method was described only by reference to another paper.]** DNA laddering was assessed based on relative fluorescence of DNA ladders on gels. Statistical analysis was by ANOVA with post hoc Dunnett test or by Kruskall-Wallis test. Litter was included as a factor in the ANOVA.

In testes evaluated on GD 21, vacuolization of Sertoli cells, shedding of gonocytes, reduced interstitial cell cytoplasm, and enlarged tubules were identified in offspring of all dams exposed to DEHP 750 mg/kg bw/day, regardless of diethylhexyl adipate co-exposure, compared to 0–14% of dams exposed to vehicle. Leydig cell hyperplasia was identified in offspring of more dams with DEHP treatment than control dams **[statistical analysis not shown]**. The number of histone H3-positive cells per testis section was not altered by treatment. **[Other immunohistochemistry results were not quantified but were not reported as affected by treatment.]** Staining for anti-Müllerian hormone to identify Sertoli cells showed positive cells within Leydig cell clusters, outside the tubules. DNA laddering was increased by DEHP treatment, although TUNEL-positive cells and caspase-3-positive cells were not increased by maternal DEHP 750 mg/kg bw/day.

On PND 26, tubules without spermatocytes were found in all litters exposed to DEHP compared to 29% of control litters **[statistical analysis not shown]**. Malformed tubules were identified in 17–29% of DEHP-exposed litters compared to none of the control litters. There were no effects of DEHP treatment on any of the measures of apoptosis on PND 22, 26, of 190, although the authors indicated that "a few animals in the treated groups had very high numbers of TUNEL positive cells, presumably spermatocytes."

The authors concluded that the development of dysgenic tubules in response to DEHP exposure was related to interstitial changes occurring during gestation, including the presence of Sertoli cells in the interstitium. They believed that Sertoli cell dysfunction in the fetal period might underlie the focal testicular dysgenesis seen in older animals. The authors proposed that the lack of alteration in Sertoli cell structure in prepubertal rats in this study might reflect recovery from DEHP, which was last administered on PND 17.

Strengths/Weaknesses: This study included a detailed description of methods, source and purity of test material, and appropriate statistical analyses, when utilized, employing nesting of offspring within litter. Animals were dosed during a relevant period of male sexual development. Both litter and individual offspring incidence for histopathology were presented in Study 1. This single-dose study at 750 mg/kg bw demonstrated clear effects on the incidence of Sertoli cell vacuolization, shedding of gonocytes, multinucleated gonocytes, and reduced cytoplasm in interstitial cells in 21 day-old rat fetuses and malformed tubules and tubules lacking sprematocytes in 26 day-old (postnatal) rat pups. This effect was clear even though statistical evaluations were not conducted on these endpoints. Study 2 explored a dose-response relationship (DEHP at 300 and 750 mg/kg bw) with 20 time-mated females/group. The number of litters/group, however, was not reported.

The use of the Wistar rat was a strength in extending the evaluation of DEHP to a strain different from that used in most other studies. Weaknesses include the small sample size (5–8 litter/group) in the first study, although effects in treated animals were observed at a high incidence and were clearly distinguishable from control. The lack of dose-response data in the first study was a weakness. The second study, which permitted dose-response evaluation, focused primarily on mechanism/mode of action and either did not present the data in a format useful for the evaluation process or included few endpoints useful for assessing risk. In addition, the dose levels used in the second study were relatively high.

Utility (Adequacy) for CERHR Evaluation Process: The single-dose study is useful in supporting previous study results but is of limited use in a quantitative evaluation. The multi-dose study is more useful for the evaluation process, although it is limited in the evaluated endpoints.

Wilson et al. (86), from the US EPA, conducted 2 in vivo studies to determine if gubernacular lesions induced by DEHP and other chemicals result from inhibition of insulin-like hormone 3 (insl3), a hormone produced by fetal Leydig cells and considered to be a marker of cell maturation. In the first study, 10 pregnant Sprague-Dawley rats were gavage dosed with 0 (corn oil vehicle) or 750 mg/kg bw/day DEHP (99% purity) on GD 14–18 (GD 1 = day sperm detected). In the second study, 3 dams/group were dosed with 0 or 1000 mg/kg bw/day DEHP on GD 14–18. The DEHP concentrations were found to induce gubernacular lesions in previous studies. Dams were killed on GD 18, and fetal testes were removed. The testes were pooled by litter for examination of insl3 expression by a polymerized chain reaction method. The second study also examined ex vivo testosterone and progesterone production by incubating the testes in media for 3 hours, measuring hormone levels in media by RIA, and pooling the data by litter. Statistical analyses were conducted on a litter basis. Analyses included ANOVA, followed by paired *t*-test if statistical significance was obtained by ANOVA. DEHP treatment significantly reduced insl3 expression by about 80% in the first study and **[~60%]** in the second study. Ex vivo testosterone production was reduced **[~50%]** in testes from the 1000 mg/kg bw/day DEHP group compared to the control group. There was no effect on ex vivo progesterone production.

The study authors proposed that DEHP intake in rodents results in delayed fetal Leydig cell maturation, which leads to reduced testosterone production and insl3 production. It was stated that reduced insl3 expression results in gubernacular malformations and undescended testis, while reduced testosterone leads to malformations in testosterone-dependent tissues.

Strengths/Weaknesses: Use of the oral route of exposure and the appropriate exposure window are strengths of this study. Statistical methods were appropriate, using the litter as the unit of analysis. The data are consistent with previous findings with evaluation of a possible mode of action for impaired testis descent. The use of a single dose level is a weakness, and the ex vivo endpoints are not directly relevant to human risk assessment.

Utility (Adequacy) for CERHR Evaluation Process: This paper has limited utility in the evaluation process.

Li et al. (87), supported by NIH, examined the effects of DEHP and 2 of its metabolites on neonatal rat gonocytes and Sertoli cells. Male Sprague-Dawley rat pups from 4–7 litters were pooled and randomly placed into groups of 4–5 pups. On PND 3 (day of birth = PND 1), the rats were gavage dosed with DEHP (> 95% purity) at 0 (corn oil vehicle), 20, 100, 200, or 500 mg/kg bw. Pups were killed 24 hours after dosing. One testis was collected and preserved in 2% glutaraldehyde for a morphological examination. The other testis was collected to examine Sertoli cell proliferation through bromodeoxyuridine (BrdU) uptake. Serum FSH was measured by RIA.

In a second experiment, Sertoli cell proliferation was measured and a morphological examination of testis was conducted at 6, 9, 12, 24, or 48 hours after rats were dosed with 0 or 200 mg/kg bw DEHP. Statistical analyses included one-way ANOVA and Student-Newman-Keuls *t*-test. In rats treated with 100–500 mg/kg bw DEHP, there was a dose-related increase in abnormally large gonocytes containing 2–4 nuclei. Multinucleated gonocytes were first detected at 12 hours following exposure to 200 mg/kg bw DEHP, and their numbers increased with time. Sertoli cell proliferation was reduced in rats treated with ≥100 mg/kg bw DEHP, as noted by significant decreases in BrdU labeling. BrdU labeling indices were reported at 27.03% in control rats, 20.83% in the 100 mg/kg bw group, 9.95% in the 200 mg/kg bw group, and 4.13% in the 500 mg/kg bw group. There was a rebound in Sertoli cell proliferation at 48 hours following treatment with 200 mg/kg bw DEHP; at that time point, the labeling index was 30.5% in the DEHP group and 24.7% in controls. Dosing with up to 500 mg/kg bw DEHP did not affect serum FSH levels.

To determine the role of DEHP metabolites, measurement of Sertoli cell proliferation and a morphological examination of testis was conducted in 4 rats/group that were gavage dosed with vehicle, 393 mg/kg bw MEHP (> 95% purity) in corn oil, or 167 mg/kg bw 2-EH (> 95% purity) in phosphate-buffered saline. The doses of MEHP and 2-ethylhexanol were equivalent to 500 mg/kg bw DEHP on a molar basis (1.28 mmol/kg bw). Like DEHP, MEHP caused an increase in large multinucleated gonocytes and a decrease in BrdU labeling. Those effects were not observed following treatment with 2-ethylhexanol.

To determine if inhibited Sertoli cell proliferation is due to altered expression of cell cycle regulators, expression of D1, D2, D3, and p27^{kipl} proteins and cyclin D2 mRNA was measured in 4–5 rats/group gavage dosed with 0, 200, or 500 mg/kg DEHP; rats were killed at 6, 8, 12, or 24 hours following dosing. Statistically significant effects included a small but reproducible decrease in cyclin D2 protein level at 8 and 12 hours following treatment with 200 mg/kg bw DEHP. **[It was not clear if protein expression was also examined in rats treated with 500 mg/kg bw DEHP.]** The decrease in D2 protein expression was confirmed by a dose-related reduction in D2 mRNA expression in the 200 and 500 mg/kg bw groups.

The conclusions of the study authors were that DEHP-induced transient reductions in Sertoli cell proliferation and changes in gonocyte morphology are mediated through MEHP, alterations in Sertoli cell proliferation do not occur as a result of changes in FSH levels, and developing testes are especially vulnerable to phthalate-induced toxicity.

Strengths/Weaknesses: An appropriate route of exposure (oral) was used, and the multiple DEHP dose groups were a strength. There was a strong dose-response with statistical significance for decreased Sertoli cell proliferation as measured by the BrdU labeling index. This study has some serious design and reporting deficiencies. Sample sizes were very small for most endpoints, 4 or 5 pups per group, with a limited dosing duration (single dose) during the postnatal period that may have been too late for many of the developmental endpoints. Pups from 4–7 litters per group were used with no control for litter effect. Multiple pups within a litter may have been assigned to a single group, potentially confounding the results. It was not clear how many pups/group were evaluated for serum FSH or if serum samples were pooled. A quantitative evaluation of FSH did not demonstrate an effect of treatment; however, the small sample size, single-dose exposure, and uncertainty regarding pooling of samples resulted in low confidence for this conclusion. There was no quantification of the pathologic findings in the testes, such as numbers of animals with specific lesions. Without incidence data or quantitative evaluations, the effect and no-observed-effect levels for most pathologic observations cannot be substantiated.

Utility (Adequacy) for CERHR Evaluation Process: Although this study utilized multiple dose levels for dose-response evaluation, the study suffered significant design and reporting

deficiencies and is not useful for the evaluation process, with the possible exception of the Sertoli cell proliferation data. Low sample size, lack of control for litter effect, single-dose exposure, and lack of quantification of pathology findings make these data unsuitable for a quantitative assessment. The primary value of these data is to characterize the known pathologic effects on the testes and provide information on mechanistism/mode of action (changes in Sertoli cell proliferation and cell cycle regulators) for these effects.

Cammack et al. *(88)*, in a GLP study commissioned by the Advanced Medical Technology Association, examined reproductive development of Sprague-Dawley rats treated iv or orally with DEHP. Beginning at 3–5 days of age, rats were treated for 21 days with DEHP (99.8% purity) at 0 (vehicle), 60, 300, or 600 mg/kg bw/day by iv infusion or 0 (vehicle) or 300 mg/kg bw/day by oral gavage. Another set of rats was dosed with 600 mg/kg bw/day DEHP by oral gavage for 19 days; this group replaced a previous group that suffered high mortality rates following gavage dosing with 1000 mg/kg bw/day. Concentration and stability of dosing solutions were verified. The dosing vehicle was Intralipid®, a 10% fat emulsion solution for iv use. Each dose group in this study consisted of 16 animals. Seven rats/group were scheduled to be killed following the dosing period, and 9/group were to be held for a recovery period until 90 days of age, at which time they were also killed and necropsied. During each necropsy period, brain, liver, spleen, heart, kidneys, and testes were weighed. A testis from each rat was fixed in Bouin solution. Histopathological examinations of testes were conducted during both necropsy periods; histopathological analyses of prostate, seminal vesicle, and epididymis were conducted only in 90-day-old animals. Sperm count, motility, and morphology were examined in the 90-day-old rats. Sperm data were evaluated by Kruskal-Wallis nonparametric ANOVA, and if there was a significant finding, a Mann-Whitney *U* test was used for pair-wise comparisons. Body weight and organ weight data were assessed by determining group differences followed by pair-wise comparisons in the case of significant findings.

The only significant body weight effect reported was reduced body weight gain in rats given 600 mg/kg bw/day by iv infusion and oral gavage. Percent changes in testes and liver weight compared to control are outlined in Table 22. **[Several organ weight effects were noted, but data were presented only for absolute liver and testes weight. Relative weights are discussed only when differences were noted from absolute weights.]** In animals killed immediately after the dosing period, absolute testis weights were significantly reduced in the 300 and 600 mg/kg bw/day oral and iv groups. **[For testis weights, CERHR calculated a BMD_{10}[2] of 122 mg/kg bw/day, a $BMDL_{10}$ of 106 mg/kg bw/day, a $BMD_{1\,SD}$ of 179 mg/kg bw/day, and a $BMDL_{1\,SD}$ of 125 mg/kg bw/day in animals treated by iv infusion. After oral exposure, the BMD_{10} was 90.7 mg/kg bw/day, the $BMDL_{10}$ was 77.4 mg/kg bw/day, the $BMD_{1\,SD}$ was 875 mg/kg bw/day, and the $BMDL_{1\,SD}$ was 628 mg/kg bw/day.]** Absolute liver weights were increased in rats given 300 or 600 mg/kg bw/day DEHP by iv infusion. **[For liver effects in iv-dosed rats, CERHR calculated a BMD_{10} of 163 mg/kg bw/day, $BMDL_{10}$ of 122 mg/kg bw/day, a $BMD_{1\,SD}$ of 101 mg/kg bw/day, and a $BMDL_{1\,SD}$ of 66 mg/kg bw/day. In orally dosed rats, the BMD_{10} was 712 mg/kg bw/day, the $BMDL_{10}$ was 196 mg/kg bw/day, the $BMD_{1\,SD}$ was 585 mg/kg bw/day, and the $BMDL_{1\,SD}$ was 193 mg/kg bw/day.]** Liver weight relative to body weight was reportedly increased in the 300 and 600 oral DEHP groups. Other absolute and relative organ weight effects reported were increased spleen weight in the iv 600 mg/kg bw/day group and decreased kidney weight in the oral 600 mg/kg bw/day group. Depletion of germinal epithelium and/or decreased seminiferous tubule diameter was noted in all animals from the 300 and 600 mg/kg bw/day oral and iv dosing groups. **[It was not clear if all 7 rats from each dose group were examined, or how many rats were affected with each type of lesion.]** Germinal epithelium depletion was rated as moderate (51–75% reduction in thickness) in the 600 mg/kg

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[2] See footnote to Table 20 for definitions and a discussion of the use of benchmark doses in this report.

bw/day oral group and mild (25–50% change) in all other dose groups given ≥300 mg/kg bw/day DEHP. Reduced tubule diameter was rated as mild (25–50% reduction in diameter) in the 600 mg/kg bw/day oral group and minimal (< 25% change) in the other dose groups treated with ≥300 mg/kg bw/day DEHP.

In animals killed at 90 days of age, testicular weights in the 300 and 600 mg/kg bw/day iv and oral groups remained lower than those of controls. **[CERHR estimated a BMD$_{10}$ of 222 mg/kg bw/day, a BMDL$_{10}$ of 190 mg/kg bw/day, a BMD$_{1 SD}$ of 169 mg/kg bw/day, and a BMDL$_{1 SD}$ of 105 mg/kg bw/day for testicular weights in the iv group. In the orally dosed group, the BMD$_{10}$ was 138 mg/kg bw/day, the BMDL$_{10}$ was 125 mg/kg bw/day, the BMD$_{1 SD}$ was 85 mg/kg bw/day, and the BMDL$_{1 SD}$ was 65 mg/kg bw/day.]** Earlier increases in absolute liver weights did not persist in 90-day-old animals. The only persisting testicular lesion, apart from reduced testis weight, was a minimal (< 25%) decrease in seminiferous tubular diameter in 2 of 5 rats in the 300 mg/kg bw/day oral group and 3 of 7 rats in the 600 mg/kg bw/day oral group. **[The number of animals examined in other dose groups was not indicated.]** No lesions were observed in prostate, epididymis, or seminal vesicles. There were no adverse effects on sperm count, motility, or morphology.

The study authors concluded that sperm and testicular histologic parameters resolved after DEHP treatment was discontinued. They stated that "lack of residual effects on sperm parameters found in this study will be important in the understanding of potential health risks from DEHP in patients undergoing critical procedures, such as ECMO in infants, and the management of these risks."

[The Expert Panel notes that an acknowledgement in this paper indicates that advice on design was received from an Expert Panel member "representing the NTP/CERHR Phthalate Expert Panel"; however, this Expert Panel member was rendering advice as an individual scientist and not as a representative of the Expert Panel.]

Strengths/Weaknesses: This GLP study was conducted using relevant routes of exposure, both iv and oral, during a relevant period of postnatal development. Multiple dose levels allowed for dose-response and benchmark dose assessment. Test material source and purity were provided. The dosing emulsification was characterized and evaluated for stability. Although the characterization and stability data for the dosing emulsification were not presented, the wording in this section suggested that DEHP concentration and stability were confirmed. This study demonstrated dose-responsive effects in a variety of parameters following both iv and oral exposure. Weaknesses included inadequate detail on statistical analyses; only sperm endpoints are addressed with specific details on analyses methods. The description of analyses of body and organ weights included reference to "standard operating procedures." The use of an alpha level of 0.01 is a weakness. The authors did not address assignment of animals to treatment or whether litter effect was controlled. The pathology incidence data were not presented, making it difficult to confirm effect levels and compare to background lesions in the control. However, even though no control data were presented, the incidence of findings was high in the treated animals and the types of lesions observed were fairly rare in untreated control animals. The variability in onset of dosing (PND 3–5) is an additiona; weakness.

Utility (Adequacy) for CERHR Evaluation Process: Portions of this study can be used for the evaluation. This study provides important dose-response information following both iv and oral exposure in young animals. The testes and liver weight and sperm assessment parameters provide quantitative multi-route dose-response data. Due to incomplete presentation of data, there is less confidence in the conclusion regarding the pathologic findings; however, pathological changes

consistent with disruption of spermatogenesis are supported by the findings of other investigators. The relatively small sample size also reduces the confidence level of these conclusions, especially with regard to lack of treatment-related effects.

Table 22. Testicular and Liver Weight Changes in Rats Treated with DEHP by IV Infusion or Oral Gavage

Weight change	Percent of control value in each dose group (mg/kg bw/day)				
	60 iv	300 iv	600 iv	300 oral	600[b] oral
Period immediately after dosing[a]					
Testis	91[a]	67***	52***	59***	36%***
Liver	99	126*	133%***	125%	108%
Following recovery period[a]					
Testis	101	88**	74***	69***	58***
Liver	98	96	89	94	89

From: Cammack et al. *(88)*.
*$P < 0.01\%$; **$P < 0.001$; ***$P < 0.001$.
[a]Rats were examined immediately after the 21-day dosing period or following a recovery period at 90 days of age.
[b]Rats in this group were dosed for 19 days, while the other dose groups were dosed for 21 days.

Liu et al. *(89)*, supported by NIH, evaluated gene expression profiles in GD-19 fetal testes after GD 12–19 gavage treatment of dams with 1 of 7 phthalates (n = 5/group) or with corn oil vehicle (n = 10; vaginal sperm = GD 0). The phthalates were DEHP or diethyl, dimethyl, dioctyl tere-, dibutyl, dipentyl, or benzyl butyl phthalate. **[Purity was said to have been verified but was not specified.]** The dose of each phthalate was 500 mg/kg bw/day. After removal by cesarean section, pups were evaluated for anogenital distance and testes were harvested from male offspring. Total RNA was extracted from the testes of 3 pups/treatment group, each from a different litter, and hybridized to a microarray gene chip. ANOVA and post hoc Dunnett test were used to evaluate differences in gene expression in phthalate-exposed and control samples. A Bonferroni adjustment was used for multiple comparisons. Selected genes were investigated further using reverse-transcriptase polymerase chain reaction (RT-PCR) on total testis RNA from 6 control fetuses and 3 phthalate-treated fetuses/group. Relative expression ratios were calculated with respect to glyceraldehyde-3-phosphate dehydrogenase and compared to control using ANOVA and post hoc Dunnett test. Comparisons between different phthalates were also evaluated using ANOVA. Immunohistochemistry was performed in formalin-fixed testicular sections from control and dibutyl phthalate-exposed fetuses **[not discussed here]**.

Anogenital distance was significantly reduced by pregnancy treatment with DEHP, dibutyl phthalate, benzyl butyl phthalate, and dipentyl phthalate. Dimethyl, diethyl, and dioctyl terephthalate did not affect anogenital distance. Of 391 significant gene probe sets, there were 167 characterized sequences. Genes related to lipid, sterol, and cholesterol homeostasis accounted for 31 of these 167 genes. There were also 10 genes involved in lipid, sterol, and cholesterol transport, 12 genes involved in steroidogenesis, 9 transcription factor genes, 22 signal transduction genes, 11 genes involved in oxidative stress, and 13 genes related to the cytoskeleton. Eighteen of these genes were evaluated using RT-PCR, and 16 of the 18 were affected by those phthalates that altered anogenital distance in comparison to the phthalates that did not alter anogenital distance (Table 23). There were few differences in relative expression between the individual phthalates in the group that altered anogenital distance. The authors concluded that the developmentally toxic phthalates were "indistinguishable in their effects on gene expression in the developing fetal testis." These phthalates were described as targeting pathways directly or indirectly related to Leydig cell production of testosterone and pathways important for Sertoli cell-gonocyte interaction.

[The Expert Panel notes that there is a study by Lehmann et al. *(90)* **in which di(n-butyl) phthalate was administered to pregnant Sprague-Dawley rats on GD 12–19 at gavage doses of 0.1, 1.0, 10, 50, 100, or 500 mg/kg bw/day. Fetal testes were evaluated for testosterone and mRNA and protein concentration for key steroidogenic enzymes. Decreases in mRNA for key steroidogenic enzymes occurred at maternal exposure levels below those associated with a decrease in testicular testosterone. To the extent that di(n-butyl phthalate) and DEHP share molecular mechanisms of action, this study may offer insights relevant to DEHP testicular toxicity.]**

Table 23. Relative Expression of Fetal Testis Genes after Phthalate Treatment of Pregnant Rats

Gene	Direction of alteration
Lipid, sterol, and cholesterol transport	
Epididymal secretory protein 1	↓
Low density lipoprotein receptor	↓
Steroidogenesis	
17β-Hydroxysteroid dehydrogenase 7	↓
17β-Hydroxysteroid dehydrogenase 3	↔
LH/chorionic gonadotropin receptor	↓
Transcription factors	
CCAAT/enhancer binding protein, beta	↓
Early growth response 1	↑
Nuclear receptor subfamily 4, group A, member 1	↑
Nuclear factor, interleukin 3, regulated	↑
Nuclear receptor subfamily 0, group B, member 1	↓
Transcription factor 1	
Signal transduction	
Insulin-induced gene 1	↓
Cytoskeleton	
Fasciculation and elongation protein	↑ *
Unclassified	
Decay-accelerating factor	↔
DOPA decarboxylase	↓
Seminal vesicle secretion 5	↓
Testis-derived transcript (testin)	↑

↑ , ↓ ,↔ Statistically increased, decreased, or unchanged on comparison of the phthalates that decreased anogenital distance (DEHP, dibutyl phthalate, benzyl butyl phthalate) with the phthalates that did not (dimethyl, diethyl, and dioctyl terephthalate).
*DEHP did not show a response, although the other anogenital distance-altering phthalates did.
From Liu et al. *(89)*.

Strengths/Weaknesses: This study was a well-conducted and reported and investigated the potential mechanisms of "testicular dysgenesis" and male reproductive tract abnormalities in rats following gestational exposure to DEHP and other phthalates. The dams were treated during a critical period of male reproductive tract development (GD 12–19) by an appropriate route (oral) of exposure. The statistical design was appropriate, using the litter as the experimental unit of analyses and incorporating body weight as a cofactor in the anogenital distance analysis. The use of both "active" and "inactive" phthalates was a strength, permitting a comparison of gene expression profiles. Weaknesses include the use of only a single dose level of 500 mg/kg bw and small sample size (5/treatment); however, the sample was sufficient to detect strong effects. The study used only 1 endpoint (anogenital distance) that is classically used for risk assessment. The remaining endpoints were mechanistic in nature (gene expression). Evaluation at a single time point is a weakness inasmuch as the noted effects could be the consequences rather than the causes of the altered differentiation.

Utility (Adequacy) for CERHR Evaluation Process: The anogenital distance data from this study are useful for hazard identification at the relatively high dose level of 500 mg/kg bw and the study supports/confirms other reports of adverse effects on this endpoint following gestational exposure to DEHP. Because only one dose level was used, a dose-response evaluation and NOAELs/LOAELs cannot be established. The gene expression assays are valuable in helping to understand potential mechanisms of action. Based on the affected genes, a complex mode of action is suggested involving Leydig cell, Sertoli cell, and Sertoli-gonocyte interactions.

3.2.1.2 In vitro exposures

Iona et al. (91), supported by "EU BIO-CT96-0183," "MURST," and the Italian Public Health Ministry, conducted in vitro studies to examine the effect of MEHP and 2 other chemicals on primordial mouse germ cells. In the first and second study, primordial germ cells were obtained from sexually undifferentiated gonadal ridges of CD-1 mouse embryos at 11.5 days post coitum and were seeded onto STO, an embryonic mouse fibroblast cell line, to allow the germ cells to proliferate. Cultures were treated with MEHP **[purity not indicated]** at 0 (dimethylsulfoxide [DMSO] vehicle), 100, 300, or 600 μM **[0, 27.8, 83.4, or 167 mg/L]** for 2 hours or 1 day. **[It was not specified if MEHP was rinsed from cells following the 2-hour and 1-day treatments.]** Numbers of primordial germ cells were counted after 1 and 3 days of culture. MEHP treatment for 2 hours did not affect numbers of primordial germ cells at any dose. Following treatment with MEHP for 1 day, there was a "slight but not significant" decrease in primordial germ cell number at 100 and 300 μM MEHP and cytotoxicity at 600 μM MEHP. It was determined that 600 μM MEHP reduced the viability of supporting STO cells by about 50%. In the third study, primordial germ cells were incubated in suspension with 100–300 μM MEHP for 2 hours. MEHP was washed from the cells, and the germ cells were then seeded onto STO cells. The numbers of primordial germ cells were measured after 1 and 3 days of culture. Numbers of germ cells were reduced by about 38% **[estimated from a graph]** at ≥300 μM MEHP **[statistical significance not indicated, but based on the graphed standard errors, the results appear to be significant]**. In a fourth study, apoptosis was not significantly increased following incubation of the primordial germ cells in 500 μM MEHP for 6 hours. In a fifth study, a short-term adhesion assay was used to determine that exposure to 300–600 μM MEHP for 2 hours reduced by about 40% the percentages of primordial germ cells capable of binding to STO cells. The study authors concluded that MEHP affected adhesion of primordial germ cells to STO cells without inhibiting growth or survival of the germ cells.

A sixth study was conducted to determine if in vitro effects of MEHP could be replicated in vivo. Pregnant CD-1 mice were gavage dosed with 1000 or 2000 mg/kg bw MEHP on day 8.5, 10.5, or 11.5 post coitum. **[One control group was used, but it was not specified if or when the control**

was gavaged.] Three mice per dose were killed 12.5 days post coitum, and gonadal ridges were taken from each embryo. Sections from at least 3 randomly selected embryos/sex were examined to determine primordial germ cell numbers. Apparent reductions in primordial germ cell numbers were noted in 3 of 5 female embryos and 2 of 5 male embryos treated with 2000 mg/kg bw DEHP 8.5 and 11.5 days post coitum, but mean differences were not statistically significant in any dose group.

Strengths/Weaknesses: A range of dose levels was used for in vitro (100–600 μM; 28–167 mg/L) and in vivo (1000–2000 mg/kg) studies, but it is a weakness that the in vitro levels were note related to in vivo exposure levels. In addition, the endpoints are not typical examples of phthalate toxicity, which limits the importance of the experiments. Although the studies were insightful, potentially useful experiments to help understand the mechanisms of action of reproductive toxicants, the in vitro studies were primarily an exercise in methods development/methods utility. The in vitro systems were not standardized or validated for their predictive value for in vivo effects (i.e., the artificial environments used may not be relevant to in vivo exposure). This opinion is supported by the authors' conclusion that their in vitro results were not always exactly predictive of the effects in vivo. The in vivo study used an insufficient number of litters, employed a single dose, and did not directly address any relevant functional outcome.

Utility (Adequacy) for CERHR Evaluation Process: This paper has no utility in a quantitative assessment, although it may provide supplemental mechanism information.

Li and Kim *(92)*, supported by NIEHS, examined the effects of MEHP on cultured fetal and neonatal rat testes during 3 periods: GD 13 (plug = GD 0), GD 18, and PND 3. GD 13 is the period when testicular cord formation and Sertoli cell differentiation begin. GD 18 represents the period when Sertoli cells are proliferating and gonocytes are mitotically quiescent. PND 3 represents the period when Sertoli cells continue proliferating and gonocytes migrate to the basal side of the seminiferous tubule and become mitotically active. Gonads were obtained from fetuses or pups of Sprague-Dawley rat dams (at least 3 dams/group and 4 offspring/dam) during each of the 3 periods of development. A testis from each animal was cultured for 3 days in medium containing MEHP (≥ 99% purity) 50, 100, or 200 μM **[13.9, 27.8, or 55.6 mg/L]** and the other in medium containing the DMSO vehicle. Assays were conducted to determine cell counts, cell differentiation and proliferation, and expression of testicular cell marker proteins. Data were analyzed by one-way ANOVA followed by pair-wise comparison. In GD 13 testes, MEHP had no effect on testicular cord formation or number of gonocytes. According to the study authors, the lack of effect on testicular cord formation meant that MEHP did not affect differentiation of indifferent gonad to testis. MEHP treatment also had no effect on expression patterns of Müllerian-inhibiting substance or GATA-4, Sertoli cell markers, or of mouse vasa homolog protein, a germ cell marker. In GD 18 testes, Sertoli cells were targets of MEHP as indicated by decreased Müllerian-inhibiting substance and GATA-4 expression and impaired Sertoli cell proliferation at ≥100 μM MEHP. Numbers of gonocytes and mouse vasa homolog protein expression were unaffected by MEHP treatment. Both Sertoli cells and gonocytes were targets of MEHP in PND 3 testes. There were significant dose-dependent reductions of Müllerian-inhibiting substance and GATA expression; Sertoli cell proliferation was inhibited at ≥100 μM MEHP. Reduced gonocyte numbers and Sertoli and germ cell disorganization were observed at ≥100 μM MEHP. Gonocytes proliferation was not inhibited, but apoptosis was increased by treatment with ≥100 μM MEHP. Seminiferous cord structure remained intact following MEHP exposure.

The study authors concluded that these results suggest that MEHP targets testicular cells during periods when they are mitotically active.

Strengths/Weaknesses: The use of multiple concentrations of MEHP allowed a dose-response evaluation. In general, the methods and results were well presented. The culture system was unique, without a history of use in other laboratories, and the absence of positive and negative controls was a weakness. The MEHP concentrations that were used were not related to in vivo exposure levels, and the assumption that exposure levels would be constant over a 3-day culture period was not verified. There were limited numbers of dams (3) and uncertain control for litter effect.

Utility (Adequacy) for CERHR Evaluation Process: The study provides evidence for a potential mode of action for adverse effects on the testes noted after in vivo embryonic or prepubertal exposure to DEHP. Because these studies were conducted in vitro, they are not directly useful in the evaluation process.

3.2.2 Developmental Studies Focusing on Non-reproductive Effects

Magliozzi et al. (93), supported by Italian "CNR" and "MURST" grants, examined the effects of prenatal DEHP exposure on rat neonatal lung. Wistar rats were fed diets containing 0 or 1% (w/w) DEHP from the week prior to delivery through 2 days following delivery. Five dams that ate at least 1000 mg DEHP/kg bw/day and 5 control dams were used for the study. Two days following delivery, pups were weighed and killed. One pup per litter was used to measure DEHP in blood by GC/MS. Lungs from 5 pups per litter were fixed in Bouin solution for examination by light microscopy, and lungs from 1 pup per litter were fixed in 4% formaldehyde for examination by electron microscopy. Lungs from 2-day-old rat pups were stated to have similar histological features as lungs from premature infants at gestation weeks 26–36. A catalase-immunoreactivity method was used to measure number and sizes of pneumocytes, cells that are a major source of surfactant and contain high numbers of peroxisomes. Pup livers were removed and weighed. **[Statistical methods were not discussed.]** DEHP in blood was measured at 4.7 ± 0.46 μg/mL **[error not specified]** in treated pups and 1.9 ± 0.57 in control pups. Relative liver weight was significantly increased in the DEHP-treated pups, thus confirming DEHP exposure. Examination of lungs by light microscopy revealed reduced respiratory surface in DEHP-treated animals as a result of fewer airspace units that were dilated and units that were less branched than spaces of untreated animals. In treated compared to control pups, relative number of type II pneumocytes increased by 187% and mean diameter increased by 120% ($P < 0.01$). Pneumocyte peroxisomes were unaffected in DEHP-treated pups. The study authors concluded that the relevancy of these results to intubated preterm infants inhaling DEHP is not known due to differences in routes of exposure and interspecies metabolism. However, they stated that their study indicates a need to examine possible injury related to forced ventilation of infants.

Strengths/Weaknesses: For most procedures, this paper included excellent presentations of methodology and results. The quantitative representation of pathology using morphometrics provided value; however, no description of the statistical analyses was noted. The investigation of a new target tissue (lung) was a strength. Weaknesses included the use of a single very high dose level without an indication that the effect is relevant to lower exposure levels. Only animals consuming at least 1 g/kg bw/day were selected, resulting in low sample size. Plasma extraction validation was conducted with human blood rather than rat blood. Significant contamination of control animals with DEHP was apparent with DEHP blood levels in controls about one-third those of treated animals. In addition, only DEHP was measured, leading to the possibility that evaluation of other analytes would have suggested even greater control animal contamination. The relatively high levels of DEHP in control pups raises serious questions regarding dosing errors and quality of study conduct and/or plasma level evaluation. Because exposure of the dams continued until the pups were killed, the pups from the DEHP-treated dams should have had

markedly higher levels of DEHP than the control pups. Methods for the statistical analyses were not presented.

Utility (Adequacy) for CERHR Evaluation Process: Although the evaluation of a new target tissue is potentially valuable, due to the deficiencies in presentation of statistical analyses and the potential contamination of the control pups, this study is of low utility.

Masuo et al. (94), from the Japanese National Institute of Advanced Industrial Science and Technology, studied the effects of DEHP and other chemicals on motor activity in rats. At 5 days following birth, male Wistar rats (~10 g) received DEHP **[purity not specified]** at 0 (olive oil vehicle) or 87 nmol **[3.4 mg/kg bw]** by intracisternal administration. Groups of 7 control pups and 5 DEHP-treated pups were nursed by lactating dams until weaning at 3 weeks of age. Spontaneous motor activity was measured in all 4-week-old pups using a Supermex activity-monitoring system. The rats were killed at 8 weeks of age. Multiple gene expression in striatum and midbrain of 3 rats/group was determined using an array, and an immunohistochemical technique was used to measure tyrosine hydroxylase levels in sectioned brains from 8-week-old rats. Statistical analyses included ANOVA, followed by post hoc Scheffé test.

DEHP caused a significant increase in spontaneous motor activity during the dark phase, light phase, and entire 24-hour period. DEHP treatment did not appear to affect stereotyped behavior. There was no effect on tyrosine hydroxylase immunoreactivity (data not shown). In midbrain, DEHP treatment up-regulated expression of genes for glutamate/aspartate transporter, superoxide dismutase 1, heat shock 90-kilodalton (kDa) protein beta, neuropeptide Y, fibroblast growth factor 10, and natriuretic peptide precursor C. DEHP treatment down-regulated midbrain expression of genes for c-fos proto-oncogene, cytochrome P450 17, heat shock 70-kDa protein 1A, dopamine receptor 1A, galanin receptor 2, arginine vasopressin receptor 2, and glutamate ionotropic receptor. In striatum, DEHP treatment down-regulated expression of genes for c-fos proto-oncogene, heat shock 70-kDa protein 1A, galanin receptor 2, glutamate ionotropic receptor, and PDGF B polypeptide. Based on analyses of gene expression changes, the study authors postulated that an inhibition of glutamatergic transmission in midbrain and striatum may be a mechanism of DEHP-induced changes in motor activity.

Strengths/Weaknesses: The hypothesis that DEHP would alter brain dopaminergic system, as has been reported for estrogenic chemicals, was reasonable. The comparison of a positive control (6-hydroxydopamine) and 4 chemicals considered capable of endocrine alterations (bisphenol A, *p*-nonylphenol, *p*-octylphenol, and DEHP), all of which caused a similar increase in diurnal spontaneous motor activity, is a strength. Weaknesses include the route of administration (intracisternal), the high dose level, the lack of information on offspring body weights, the apparent lack of control for litter effects, the small sample sizes (5–7 litters), and the use of a single dose level. It is not clear to what extent DEHP would be metabolized after intracisternal administration.

Utility (Adequacy) for CERHR Evaluation Process: This study is not useful in the evaluation process.

Tanaka (95), support not indicated, examined neurobehavioral toxicity in mice exposed to DEHP (> 97% purity) during prenatal development. At 5 weeks of age, 10 CD-1 mice/sex/group were fed diets containing 0, 0.01, 0.03, or 0.09% DEHP for 4 weeks prior to mating and during a 5-day mating period that began at 9 weeks of age. Females continued to receive the control or DEHP-containing diets throughout the gestation and lactation periods. The authors converted DEHP doses to a mg/kg bw/day basis, and those values are summarized in Table 24. Each female was mated to 1 male, and the females were allowed to litter and rear their offspring. At birth (PND 0),

litter size, litter weight, and sex ratio were determined. Offspring were individually weighed, and postnatal survival was monitored during the lactation period. Neurobehavioral parameters examined in all offspring during the lactation period included surface righting (PND 4 and 7), negative geotaxis (PND 4 and 7), cliff avoidance (PND 7), swimming behavior (PND 4 and 14), and olfactory orientation (PND 14). Weaning occurred at 4 weeks of age, and 1 male and 1 female per litter were selected to continue receiving treatment until 9 weeks of age. **[Though not specified, it is assumed that the offspring from each treatment group received the same doses as their parents.]** Doses on a mg/kg bw/day basis for offspring are also included in Table 24. At 7 weeks of age, the F_1 mice were tested using a Biel type water T-maze. Exploratory behavior was assessed using an animal movement analyzing system in 3-week-old mice from the F_1 generation and 8-week-old mice from the F_0 and F_1 generations. Statistical analyses included Bonferroni multiple comparison, ANOVA, Kruskall-Wallis test, chi-squared test, Fisher exact test, Sign-Wilcoxon test, and/or Jonckheere test. **[It does not appear that statistical analyses were conducted on a per litter basis.]**

In F_0 mice, DEHP treatment had no effect on body weight gain, movement, or exploratory activity. As a result of non dose-related failures to become pregnant or abortions in 1–2 dams of the low- and mid-dose groups, 8–10 litters were available for evaluation in each treatment group. There were no significant effects on sex ratio or litter size or weight at birth. A 7% decrease in body weight in male offspring of the low-dose group compared to control males on PND 0 was the only significant body weight effect observed in offspring. Significant reductions in survival were noted in the high-dose group for female offspring from PND 4 to 14 and for total offspring from PND 4 to 21. Percentages of total surviving offspring at PND 21 were 98.4% in the control group and 92.8% in the high-dose group. Time for surface righting was significantly delayed in females of the low- and mid-dose groups on PND 4, in males of the high-dose group on PND 7, and in females of the low-dose group on PND 7. There were no other significant findings in neurobehavioral parameters examined during the lactation period **[data not shown]**. Compared to controls, there were no adverse effects in water T-maze performance in treated animals at 7 weeks of age, and movement and exploratory behavior were not affected by treatment at 3 or 8 weeks of age. The study authors concluded that "few adverse effects on several behavioral parameters were produced at the high-dose level of DEHP in the present study."

Table 24. Summary of DEHP Doses in Mice

Generation and study period	Mean DEHP doses, mg/kg bw/day[a], by diet group		
Diet group:	0.01%	0.03%	0.09%
F_0 males premating	16	47	142
F_0 females premating	20	56	168
mating	15	40	126
gestation	17	47	140
lactation	60	172	493
F_1 males	16	48	145
F_1 females	19	56	171

From: Tanaka *(95)*.
[a]Values were presented as mean ± SD by study authors; the values presented here are means rounded to whole numbers because that information is sufficient for the CERHR evaluation process.

Strengths/Weaknesses: Source and purity of test material were provided and an appropriate route of exposure (diet) was used. The use of relatively low dietary exposure levels was a strength. Additional strengths included the use of multiple dose levels and multigenerational exposure in mice from 5 weeks of age for the starting F_0 generation through 9 weeks of age for the F_1 generation, encompassing the premating, gestation, lactation, and sexual maturation

periods. For the F_0 generation animals, the post-weaning evaluations were controlled for litter effect by selecting one male or female per litter. Litter means were also used in the evaluation of pup weight and litter size. It is unfortunate that animals were not evaluated for the classic phenotype of prenatal phthalate exposure, which would have extended the observations to another species. Sexually dimorphic behaviors should have been evaluated, given the presumed mode of action involving a reduction in fetal steroid hormone levels. There are weaknesses in the presentation and analysis of data. The preweaning data for surface righting, negative geotaxis, cliff avoidance, swimming behavior, and olfactory orientation were analyzed on an individual animal basis and were not controlled for litter effect. Of these endpoints, the authors only presented data for surface righting. Exploratory behavior data at 8 weeks of age were also not presented. Preweaning survival indices were also not analyzed on a litter basis. There was a limited set of parameters evaluated; endpoints of sexual development, fertility, and pathology were not evaluated. Sample size was limited for this type of experiment.

There are questions concerning study conduct, detail of methods, and data interpretation that cast doubt on the reliability of this report. It appears that a number of the behavioral tests were performed on the same day (surface righting, negative geotaxis and cliff avoidance on PND 7, surface righting, negative geotaxis and swimming behavior on PND 4, swimming behavior and olfactory orientation on PND 14). There is no mention of order of testing or time of testing, both of which are important for data interpretation. For example, swimming behavior was tested on PND 4 and 14 in water at 23ºC, which is quite cold, especially for such young animals. There is no mention of how these animals were handled afterwards, specifically whether they were put under a heat lamp to normalize body temperature. The possible body temperature alterations could have affected surface righting and negative geotaxis on PND 4 if these tests were performed after the swimming trial. It is not clear if all of these tests were conducted in the same room, which could impact interpretation based on general activity in room. The PND 4 data indicate that almost 80% of mice were swimming in a circular fashion with about 20% swimming in a straight line. This performance does not appear consistent with experience using rats, which are better swimmers than mice and which do not swim in a straight line until much later in development. The mice in this study also made far fewer errors in the T-maze than would be expected using rats, and control mice failed to demonstrate learning with repeated exposure to the maze.

Utility (Adequacy) for CERHR Evaluation Process: This study has limited utility for the specific endpoints for which data were presented and litter effect was controlled (litter size, average pup weight, and T-maze performance). None of these endpoints demonstrated a significant dose-responsive treatment effect; however, there is low confidence in these results due to use of relatively small group sizes (8–10 litters/group). Other endpoints from the F_1 offspring are not useful due to deficiencies in statistical evaluation and/or lack of data presentation.

James (96) wrote a letter to the editor to question Tanaka's (95) conclusion that sex ratios were not affected in offspring of mice fed diets containing 0.01–0.09% DEHP during pregnancy. James noted that there were a total of 190 male offspring and 152 female offspring in all DEHP treatment groups and that the difference in sex ratio was significantly higher than equality (*P* <0.05). It was stated that DEHP can have opposite effects on endocrine parameters (e.g., testosterone and estradiol levels) in male versus female rats, and that it could potentially affect offspring sex ratio differently in treated males versus females. Because Tanaka mated treated female mice with treated male mice, James concluded that lack of effect of DEHP on sex ratio could not be ruled out. A later study by Tanaka (97) did examine sex ratios in DEHP-treated male and female mice mated with untreated control animals and that study is addressed below. **[The James letter is noted for completeness but is not used in the evaluation process.]**

Tanaka *(98)* responded to the James letter *(96)* regarding sex ratios in the Tanaka *(95)* study. Tanaka re-analyzed the data on a litter basis using Steel multiple comparison test and demonstrated there were no significant differences in sex ratios of offspring from DEHP-treated versus control mice. It was noted that variations in sex ratio were much higher among litters than among treatment groups. Tanaka noted that other studies in rodents also failed to demonstrate an effect on sex ratio following DEHP treatment. Lastly, Tanaka stated that sex ratios in all treated groups were within ranges observed in control mice in his laboratory during the past 10 years. Tanaka concluded that there was no experimental evidence that DEHP adversely affects sex ratio in offspring of DEHP-treated mice.

Strengths/Weaknesses: This re-evaluation of data from the previous study used a more appropriate litter-based analysis. The re-analysis and discussion of additional studies referenced in the literature and historical control data from the author's laboratory fully support the lack of an effect on sex ratio following exposure (as studied) in mice.

Utility (Adequacy) for CERHR Evaluation Process: This information supports previous conclusions *(95)*.

Tanaka *(97)*, support not indicated, examined the effects of prenatal DEHP exposure on sex ratio in mice. It appears that the study was conducted to address concerns about DEHP effects on sex ratio that were raised in letter by James *(96)*. Starting at 5 weeks of age, 20 male and female CD-1 mice/sex/group were fed diets containing 0 or 0.03% DEHP (purity > 97.0%). At 9 weeks of age, each female was mated for 5 days with a male from the same or opposite treatment group (i.e., cross-mating). There were 4 treatment groups consisting of 10 mice/sex: control females × control males, control females × treated males, treated females × control males, and treated females × treated males. Females continued to receive the DEHP-containing or control diets during the mating period and throughout gestation. The study authors estimated that intake of DEHP was ~47–49 mg/kg bw/day in males and ~55–58 mg/kg bw/day in females during the preconception period. Intakes by females were estimated at ~45 mg/kg bw/day during the mating period and ~50 mg/kg bw/day during the gestation period. Females were allowed to litter, and endpoints examined on day of birth were litter size, litter weight, individual offspring weight, and sex ratio. Statistical analyses included ANOVA or Kruskal-Wallis test followed by Bonferroni multiple comparison to assess food intake, litter size, and litter and body weights. Chi-squared test was used to evaluate sex ratio based on offspring, and the Steel test was used to assess sex ratio based on litter. As a result of pregnancy failures or abortions, there were 8–10 litters delivered in each treatment group. Compared to the group consisting of control males and females, mean body weights of male offspring were increased in all groups containing a treated female and/or male parent. No significant effects were noted for litter size, litter weight, total or average sex ratio, or female offspring weights. The study authors concluded that the concentrations of DEHP used in this study did not produce adverse effects on sex ratios.

Strengths/Weaknesses: Source and purity of test material was provided, and an appropriate route of exposure (diet) was used. Exposures were conducted during the critical period of sexual differentiation. Mice were exposed from 5 weeks of age from the start of the F_0 generation through delivery of the F_1 offspring, encompassing the premating and gestation periods. It is a strength that a factorial design was used based on exposure of one sex bred with an unexposed or exposed mate and that litter was the unit of analyses for most reproductive and developmental endpoints evaluated. It is a weakness that only a single dose level was used (0.03% in the diet) and that classical measures of phthalate toxicity in male offspring were not evaluated. Sample size was limited (8–10 litters/group) for this type of experiment, resulting in low confidence in

negative outcomes or marginal effects. For example, statistically significant increases in male offspring weight were observed in all groups with DEHP parents. However, in two other studies by this author utilizing a similar study design exposing mice to equal or higher doses of DEHP, no effect on pup weights or an increase in female pup weight was found. (The author concluded that the male pup weights in the concurrent control were unusually low and the effect of increased male pup weight was not treatment-related. The Expert Panel agrees).

Utility (Adequacy) for CERHR Evaluation Process: This study is limited in scope to litter size, litter weight, pup weight and sex ratio, and to a single dose level of 0.03% in the diet (40–56 mg/kg/day).

Tanaka et al. *(99 332)*, support not indicated, gave DEHP (>97% purity) to CD-1 mice in the diet from 5 weeks of age in the F_0 generation to 9 weeks of age in the F_1 generation. A single dietary dose level of 0.03% was used, with control animals receiving untreated basal feed (n = 20/sex/treatment group). At 9 weeks of age, 10 DEHP-treated females were paired with DEHP-treated males, 10 DEHP-treated females were paired with control males, 10 control females were paired with DEHP-treated males, and 10 control females were paired with control males. The females' diet was available to males during the 5-day cohabitation phase. Females reared their own unadjusted litters, which were weaned at 4 weeks of age. One female and male from each litter were retained and fed their dam's diet until 9 weeks of age.

All F_1 offspring underwent neurobehavioral testing during the lactation period, including surface righting and negative geotaxis on PND 4 and 7, cliff avoidance on PND 7, swimming behavior on PND 4 and 14, and olfactory orientation on PND 14. Exploratory behavior was assessed in 1 male and 1 female from each litter at 3 weeks of age. Post-weaning tests included multiple-T water maze at 7 weeks of age and exploratory behavior at 8 weeks of age. Statistical analyses were performed using ANOVA or Kruskal-Wallis test followed by Bonferroni multiple comparison test. Proportions were evaluated using chi-squared or Fisher test. **[It is not stated whether litter was considered in the analysis of the preweaning neurobehavioral tests.]**

Based on measured feed consumption, mean DEHP intake by treated males **[rounded by CERHR]** was 46 mg/kg bw/day. Treated females received 53–57 mg/kg bw/day during the preconception period, ~43 mg/kg bw/day during mating, 46–49 mg/kg bw/day during gestation, and 154–171 mg/kg bw/day during lactation. DEHP had no effect on feed consumption or dam body weight. As repeated in Section 4.2.3, there were no significant treatment effects on the number of pregnant females, number of litters, number of offspring, average litter size or weight, or offspring sex ratio. Offspring body weight during the lactation period was similar between groups except for an 8% decrease in body weight on PND 14 in female offspring when the parents both had received DEHP. The author did not consider this isolated alteration to be treatment related. Swimming ability was accelerated in PND 4 female offspring when the dam received DEHP. The number of movements in the test of exploratory behavior was decreased in male offspring the parents of which both received DEHP. There were isolated differences in T-maze performance by sex, trial, and treatment group that were not considered to represent treatment-related alterations in maze-learning. None of the other behavioral tests revealed effects of DEHP treatment.

The author concluded that "few adverse effects on several behavioral parameters were produced at the dose level of DEHP in the present study."

Strengths/Weaknesses: Source and purity of test material were provided, and an appropriate route of exposure (diet) was used. The use of multigenerational exposure in mice from 5 weeks of

age for the F_0 generation through 9 weeks of age for the F_1 generation encompassed the premating, gestation, lactation, and sexual maturation periods. The use of factorial design based on exposure of one sex bred with an unexposed or exposed mate was a strength. For the F_0 generation animals, the post-weaning evaluations were controlled for litter effect by selecting one male or female per litter. Litter means were also used in the evaluation of pup weight and litter size. It is a weakness that only a single dose level was used, although it is a strength that this dose level was relatively low. Sample size was limited (8–10 litters/group), resulting in low confidence in negative outcomes. For example, the author states that no effect was observed on pup weight, with the exception of decreased female pup weights on PND 14 in group 4. However, the data suggest that decreased weight in both sexes at all preweaning time points would have been evident had a sufficient sample size been used. Sexually dimorphic behaviors were not evaluated. Other weaknesses in the presentation of data and conduct of behavioral testing are as discussed above for Tanaka *(95)*.

Utility (Adequacy) for CERHR Evaluation Process: This study has limited use only for specific endpoints in which data were presented and litter effect controlled (litter size, mean pup weight, post-weaning exploratory behavior, and T-maze performance). None of these endpoints demonstrated a significant dose-responsive treatment effect; however, there is low confidence in these results due to use of a relatively small group sizes (8–10 litters/group). All other endpoints on the F_1 offspring are not useful for the evaluation due to deficiencies in statistical evaluation and/or lack of data presentation.

Lee et al. *(100)*, supported by the South Korean Ministry of Environment, examined the expression of zinc-metabolizing enzymes in mouse dams and embryos exposed to DEHP. One hypothesis is that altered zinc homeostasis is a cause of teratogenicity following DEHP exposure. On GD 9 (9 days post-coitus), CD-1 mice were given corn oil (vehicle) or DEHP 800 mg/kg bw by gavage. Dams were killed at 3, 4.5, or 6 hours following exposure, and maternal liver, visceral yolk sac, and embryonic forebrain were collected. Polymerase chain reaction and Western blotting techniques were used to study expression of zinc-metabolizing enzymes in the collected tissues. Results were analyzed by Student *t*-test. Maternal liver expression of metallothionein (MT)-I and MT-II, enzymes that sequester zinc in liver and thus lower blood levels, were increased at 3.0–4.5 hours following DEHP exposure and then began returning to baseline levels at 6 hours following exposure. Maternal liver expression of zinc transporter-1 (ZnT-1), a transmembrane protein involved in zinc efflux, was not affected by DEHP exposure. Exposure to DEHP resulted in a down-regulation of MT-I, MT-II, and ZnT-1 expression in embryonic brain from 3–6 hours following exposure. There was no effect on visceral yolk sac.

A dose-response study was conducted in which pregnant mice were gavaged on GD 9 with 0, 50, 200, or 800 mg DEHP/kg bw. Dams were killed, and maternal liver and embryonic brains were collected at 3 hours following exposure. According to the text of the study, up-regulation of MT-I and MT-II in maternal liver reached statistical significance at 200 mg/kg bw/day DEHP. **[Table 1 of the study indicates that the increase in MT-II expression reached statistical significance at 800 mg/kg bw.]** The study authors calculated a BMD_5 of 6.7 mg/kg bw for MT-1 and 5.6 mg/kg bw for MT-II. $BMDL_5$s (lower 95% confidence limit) were calculated at 3.7 mg/kg bw for MT-I and 3.2 mg/kg bw for MT-II. In embryonic brain, reductions in MT-I and ZnT-1 were significant at 200 mg/kg bw, and reductions in MT-II were significant at 50 mg/kg bw. Study authors calculated BMD_5 responses of 11.6 mg/kg bw for MT-I, 8.9 mg/kg bw for MT-II, and 6.6 mg/kg bw for ZnT-1. $BMDL_5$s (lower 95% confidence limit) were calculated at 7.1 mg/kg bw for MT-I, 5.2 mg/kg bw for MT-II, and 3.9 mg/kg bw for ZnT-1. The study authors concluded that exposure of dams to DEHP during periods of organogenesis can alter the expression of key fetal enzymes involved in zinc homeostasis.

Strengths/Weaknesses: This paper is based on a strong hypothesis and includes good presentation of methods and results. Multiple dose levels were evaluated and a dose-response was demonstrated. The paper lacked direct measurement of zinc levels in sensitive tissue to correlate with the gene expression/protein data. MT-I/MT-II and ZnT-1 are not validated biomarkers for the endpoints of concern (affected by DEHP). Changes in gene expression alone are not adverse, may be adaptive, and must be linked directly to an adverse outcome, which was not done. In addition, even after direct linkage to adverse outcome, the threshold of change in gene expression in order to induce an adverse effect should be established. Much additional work needs to be completed before changes in gene expression and protein levels can be used as surrogates for adverse outcomes (e.g., direct measurement of zinc levels in sensitive tissue and correlation with adverse outcome and gene expression, consistency across multiple tissues affected, zinc supplementation rescue). Finally, the authors conclude "How this mechanism contributes to the overall developmental toxicity of phthalates in general and DEHP in particular remains to be further examined." This statement clearly indicates the authors believe their information is useful for hypotheses generation, preliminary in nature, and in need of further development before it can be used directly in risk assessment. An additional limitation is the time of dosing, which is not the most critical period for phthalate adverse effects on male reproductive organ development.

Utility (Adequacy) for CERHR Evaluation Process: This paper is not directly useful in the evaluation process, although it provides good hypothesis-generating information for explaining potential mechanisms.

Rhee et al. (*101*), supported by the Korean Food and Drug Administration, evaluated the developmental effects of DEHP using in vitro tests. Whole embryo culture was performed by explanting GD 9.5 Wistar rat embryos into serum-based media treated with DEHP **[purity not specified]** in 0.5% DMSO. DEHP concentrations were 1, 10, or 100 µg/mL **[mg/L],** n = 15–28 embryos/concentration. Comparisons were made to untreated (n = 35 embryos) and DMSO vehicle-treated (n = 30 embryos) controls. Cultures were maintained for 48 hours, following which embryos were evaluated for yolk sac diameter, crown-rump length, head length, somite number, and Maele-Fabry morphology score. Data were analyzed using ANOVA with post hoc Bonferroni or Dunnett test. Yolk sac diameter, somite number, and Maele-Fabry score were significantly decreased at all concentrations of DEHP. Crown-rump and head length were decreased by the 2 highest concentrations of DEHP. The authors also performed micromass cultures using dissociated limb bud and midbrain cells from GD 12.5 embryos. Cell suspensions were allowed to attach for 2 hours, following which they were exposed to DEHP at concentrations ranging from 7.81 to 1000 µg/mL for 96 hours. Cytotoxicity was determined using neutral red. Differentiation was determined in limb bud cells using Alcian blue uptake and in midbrain cells using hematoxylin staining followed by quantification of differentiated foci with an image analyzer. The planned endpoint was a comparison of the concentrations at which differentiation and cell survival were 50% inhibited compared to a control value. The authors reported that the planned inhibitory concentration comparison could not be carried out. **[The authors do not give a reason for not being able to report a result, but inspection of a graph in the paper suggests that a 50% effect was not achieved for both cytotoxicity and differentiation in either the limb or midbrain culture system.]** The authors concluded that the absence of evaluable effect in the micromass assay in the face of prominent effects in the whole embryo culture system was consistent with lack of metabolism of DEHP to toxic intermediate(s) in the cell culture system.

Strengths/Weaknesses: The use of multiple in vitro screening methods and multiple exposure levels are strengths. Comparison to other phthalates was a strength, but failure to include

"inactive" phthalates was a weakness. The assays are used for potential screening and mechanistic studies, however, rather than for risk assessment. No mention was made of how the embryos were assigned to groups and whether litter effect was controlled. There was no analytical support for metabolite formation in culture media to support the hypothesis on toxic metabolite formation in the whole embryo culture.

Utility (Adequacy) for CERHR Evaluation Process: This paper is not useful in the evaluation process.

3.2.4 Fish

Chikae et al. (102), support not indicated, evaluated the hatching, survival, and sex ratio of Japanese medaka (*Oryzias latipes*) exposed to DEHP **[purity not given]** in ethanol at nominal DEHP concentrations of 0, 0.01, 0.1, 1.0, and 10.0 μg/L. Final ethanol concentrations were <100 μg/L. Fish were exposed during the "embryo stage" **[exact time of exposure not indicated]**. Observations were made of hatching time, hatching success, mortality, body weight **[age not specified, but possibly at 5–6 months, when the experiment was terminated]**, sex ratio, and gonadosomic index **[not defined; other authors have used this term for relative gonad weight]**. There were significant delays in hatching among the eggs exposed to DEHP 0.1 and 1.0 μg/L but not among the eggs exposed to DEHP 10.0 μg/L. The percent of embryos showing eye development and the percent successfully hatching were not altered by DEHP exposure. Adult mortality was increased in the groups exposed to DEHP 0.01, 0.1, and 1 μg/L but not in the group exposed to DEHP 10.0 μg/L. Sex ratio was reduced (fewer males) in the 0.01 μg/L group only. Body weight was reduced in a DEHP concentration-dependent manner with significant difference from control at and above 0.1 μg/L. The authors concluded that DEHP exposure of Japanese medaka embryos "negatively affected some biological parameter[s] in both embryo and adulthood." They acknowledged that the effects were not necessarily dose-dependent.

Strengths/Weaknesses: Multiple exposure levels allowed for a dose-response evaluation. The quantitative presentation of data was fairly good. There were clear effects on some endpoints, but changes in many parameters appear random, unrelated to dose. Findings were not interpreted in light of postulated mode of action in the development of mammalian males. Evaluation of embryo levels of the active fish androgen (11-ketotestosterone) would have been helpful.

Utility (Adequacy) for CERHR Evaluation Process: This paper is not useful in the evaluation process.

3.2.5 Abstracts

CERHR retrieved several abstracts reporting developmental toxicity associated with DEHP exposure. Although information from abstracts is not considered by the Expert Panel in reaching final conclusions, the abstracts are briefly summarized for the sake of completeness.

Borch et al. *(103)* gavage dosed pregnant Wistar rats on GD 7–21 with DEHP 0 or 750 mg/kg bw/day or with a combination of DEHP 750 mg/kg bw/day plus di(2-ethylhexyl)adipate 400 mg/kg bw/day. Testosterone production by offspring testes over a 3-hour ex vivo incubation period was reduced from 2.9 ng in controls to 0.4 ng in the DEHP-exposed group and to 0.9 ng in the group exposed to DEHP plus di(2-ethylhexyl)adipate. Testicular testosterone was reduced from 1.8 ng/testis in controls to 0.4 ng/testis in the group exposed to DEHP and 0.3 ng/testis in the group exposed to DEHP plus di(2-ethylhexyl)adipate. Plasma testosterone was reduced in male offspring from 188 pg/mL in controls to 25 pg/mL in the group exposed to DEHP and 18 pg/mL in the group exposed to DEHP plus di(2-ethylhexyl)adipate. There were no significant differences between the effects of DEHP alone and DEHP plus di(2-ethylhexyl)adipate. Similar

treatment of dams during gestation and lactation resulted in no significant alterations in serum testosterone in male offspring evaluated on PND 27.

Phokha et al. *(104)* evaluated the toxicokinetics of DEHP and MEHP in pregnant and nonpregnant Sprague-Dawley rats. Nearly all orally administered DEHP was hydrolyzed in the intestine to MEHP. The AUC of DEHP was 0.7% that of MEHP. The AUC did not change in nonpregnant rats with repeated administration and was 950, 970, and 1070 nmol-h/L per mmol/kg dose on the first, fourth, and seventh consecutive days of treatment. In pregnant rats, administration on GD 14–19 resulted in AUC values (nmol-h/L per mmol/kg dose) of 1250 on GD 14 and 970 on GD 19.

Foster and Barlow *(105)* gavage dosed 10 pregnant Sprague Dawley rats per group on GD 12–21 with corn oil vehicle, 100 mg/kg bw/day DEHP, 100 mg/kg bw/day dibutyl phthalate, or 100 mg/kg bw/day DEHP in combination with 100 mg/kg bw/day dibutyl phthalate. Treatment with DEHP + dibutyl phthalate reduced anogenital distance on PND 1 and increased retained areolae on PND 13 in male offspring; there were no significant effects in rats treated with either phthalate alone. Only a small number of reproductive organ lesions were observed in the phthalate groups. There were no consistent changes in organ weights. The study authors concluded that the effects of the 2 phthalates were additive.

Lambright et al. *(106)* treated pregnant Sprague-Dawley rats with DEHP 1000 mg/kg bw/day on GD 14–18. Fetal testes were harvested on GD 18 and placed in culture for 3 hours. Testosterone and progesterone production in culture were decreased by gestational DEHP treatment.

Gray et al. *(107)* gavage dosed Sprague-Dawley rats with DEHP at 0, 11, 33, 100, or 300 mg/kg bw/day from GD 8 to PND 17. Dosing continued in half of the male offspring from PND 18 to 63–65, at which time they were necropsied. In those offspring necropsied on PND 63–65, liver and adrenal weights were affected at ≥11 mg/kg bw/day, puberty was affected at ≥100 mg/kg bw/day, and reproductive organ weights were affected at 300 mg/kg bw/day DEHP **[no further details were provided regarding effects]**. The other half of male offspring were not dosed further after PND 17 and were necropsied upon reaching full maturity. Permanent effects noted in those offspring treated with 300 mg/kg bw/day DEHP were reductions in anogenital distance, reduced reproductive organ weights, and increased incidence of nipples. Testicular and/or epididymal abnormalities were observed in 25% of offspring from the 300 mg/kg bw/day group; a low incidence of malformations was observed at the lower dose levels.

Gray et al. *(108)* treated pregnant and lactating Sprague-Dawley rats with DEHP from GD 8 to day 17 of lactation at dose levels of 0, 11, 33, 100, or 300 mg/kg bw/day, given by gavage. Dosing was continued in 2 or 3 males/litter from PND 18 until PND 63–65. Puberty was delayed and males were heavier at the time of preputial separation in the 100 and 300 mg/kg bw/day groups. The authors concluded that DEHP is similar to dibutyl and benzyl butyl phthalate in exerting anti-androgenic effects on the developing androgen-signaling pathway.

Wilson et al. *(109)* treated pregnant Sprague-Dawley rats with oral DEHP 0, 100, 300, 600, or 900 mg/kg bw/day on GD 8–18. GD-18 fetal testes were harvested and incubated for 3 hours in 500 μL medium, following which media were evaluated for hormone concentrations. Harvested testicular were investigated using RT-PCR for assessment of gene expression. DEHP treatment resulted in a decrease in testicular testosterone production in the 300, 600, and 900 mg/kg bw/day groups and a decrease in testicular progesterone production in the 900 mg/kg bw/day group. Insl3 gene expression was decreased in a dose-dependent manner.

Hass et al. *(110)* treated pregnant and lactating Sprague-Dawley rats (n = 8/group) with gavage doses of DEHP at 0, 10, 30, 100, 300, 600, and 900 mg/kg bw/day from from GD 7 to PND 17. Anogenital distance was decreased and nipple retention increased at all DEHP doses compared to the control, although the anogenital distance difference from control was not statistically significant in the groups exposed to less than 300 mg/kg bw/day. The authors suggested that nipple retention may be a more sensitive indicator of anti-androgenic effects on development.

Numtip et al. *(111)* evaluated the toxicokinetics of DEHP in pregnant (n=4) and nonpregnant (n=8) marmosets. Animals were treated orally with 30 or 500 mg/kg bw/day for 29 days (GD 96–124). The AUCs for DEHP were about 20 times less than the AUCs for MEHP. The concentration-time curve for MEHP showed an oscillating pattern that the authors concluded represented enterohepatic cycling of MEHP glucuronide, which is not encountered in rats. Pregnancy did not alter the AUC of MEHP after the low dose of DEHP, but some reduction in MEHP AUC occurred in late pregnancy at the high DEHP dose. Maximum MEHP concentrations and AUCs were lower in pregnant marmosets than has been reported for pregnant rats.

Regnier *(112)* incubated GD 9 rat embryos for 48 hours in serum obtained from CD rats treated with 1000 mg/kg bw DEHP on GD 6–11. The DEHP-exposed embryos had increased malformations and reduced crown-rump and head lengths, somite numbers, and morphological scores. In a second in vitro experiment comparing embryotoxicity of DEHP and its metabolites, the order of potency (highest to lowest) was MEHP oxidized metabolites > DEHP = MEHP = 2-ethylhexanol > 2-ethylhexanoic acid.

3.3 Utility of Developmental Toxicity Data

Since the initial CERHR evaluation of DEHP, 2 human studies were published that examined associations between in vivo DEHP or metabolite levels and adverse development outcome or premature thelarche. Another human study examined puberty in children who had received ECMO as neonates. Six multiple dose-level animal toxicity studies have been published since the original DEHP evaluation. The studies focused on the effects of gestational or neonatal DEHP exposure on reproductive organ toxicity, pulmonary toxicity, and neurobehavioral endpoints. One study compared testicular toxicity following neonatal exposure to DEHP through the oral or iv route. Numerous studies focused on mechanistic aspects of DEHP-induced toxicity, such as effects on testosterone production, identification of target cells, interaction with the androgen receptor, and effects on zinc metabolism.

3.4 Summary of Developmental Toxicity Data

3.4.1 Human Data

In a study conducted in Italy, Latini et al. *(72)* analyzed the relationships between DEHP and MEHP in cord blood and adverse neonatal outcomes. DEHP and/or MEHP were detected in 74 of 84 cord blood samples. Mean cord blood serum concentrations were 1.19 μg/mL (range 0–4.71 μg/mL) for DEHP and 0.52 μg/mL (0–2.94 μg/mL) for MEHP. Mean gestational age was significantly lower in MEHP-positive neonates (38.16 ± 2.34 [SD] weeks) versus MEHP-negative neonates (39.35 ± 1.35 weeks) (P = 0.033). Logistic regression analyses demonstrated a positive correlation between lack of MEHP in cord blood and gestational age at delivery (OR 1.50, 95% CI 1.013–2.21). There were no significant associations between DEHP or MEHP concentrations and infant sex, delivery mode, maternal smoking, premature membrane rupture, cord loops, neonatal jaundice, small infant size, birth weight, 1- or 5-minute APGAR scores, or maternal age.

Rais-Bahrami et al. *(74)* examined onset of puberty and sexual maturity parameters in 14–16-year-old adolescents (13 males and 6 females) who had been potentially exposed to high DEHP levels as a result of receiving ECMO as neonates. Except for 1 female with Marfan syndrome, growth percentiles were normal for age and sex. Pubertal development was stated to be normal. **[Although it was stated that testicular volume and phallic length were measured, the results were not discussed by study authors.]** Laboratory results indicated normal thyroid, liver, and renal function. LH, FSH, testosterone, and estradiol levels were normal for stage of pubertal development. No control children were evaluated.

Swan et al. *(73)* measured MEHP and its oxidative metabolites in the urine of pregnant women. Anogenital distance was evaluated at 2–18 months of age in the male children born to these women. There was no significant association between maternal urinary MEHP concentration and infant anogenital distance, although the authors noted that the regression coefficients for 5-oxo- and 5-hydroxy-MEHP were of similar magnitude to regression coefficients for phthalate monomers that were significantly associated with reduced anogenital distance.

3.4.2 Experimental animal data

Experimental animal studies using multiple dose levels and thus providing dose-response information are summarized in Table 25.

In a number of studies, developing rats were exposed to DEHP and subsequently examined for effects on reproductive and endocrine systems. Three studies were notable, 2 that examined dose-related effects of DEHP exposure, and a third that compared testicular toxicity in rats dosed by the oral versus iv route. The remaining studies largely focused on mechanisms of developmental reproductive toxicity.

Moore et al. *(81)* orally dosed at least 8 pregnant Sprague-Dawley rats/group with DEHP at 0, 375, 750, or 1500 mg/kg bw/day from GD 3 (GD 1 = day after sperm detected) to PND 21. Parameters associated with sexual development were observed through puberty or adulthood in male and female offspring, and male offspring were tested for sexual behavior. DEHP treatment reduced prenatal maternal weight gain at the middle and high dose. There was no significant effect on implantation sites. Number of pups born was reduced at the high dose, and postnatal survival was decreased at the middle and high dose. The most sensitive DEHP effect on males was an increase in areolas or nipples, which occurred at all dose levels and persisted through adulthood. Incomplete preputial separation, non-descent of testes, and agenesis of anterior prostate and other accessory reproductive organs did not attain statistical significance at the lowest dose level, but the authors considered them to be biologically significant at all doses due to the rarity of the effects. Reproductive effects observed in males exposed to higher doses included reduced anogenital distance, agenesis of seminal vesicles and epididymides, decreased sperm counts, and reduced weights of testes, epididymides, glans penis, prostate, and seminal vesicles that often persisted through adulthood. Though statistical significance was not attained, presumably due to use of small numbers of animals, sexual behavior tests were said to show adverse effects in all dose groups as characterized by fewer mounts, intromissions, and/or ejaculations. In female pups, DEHP treatment had no effect on anogenital distance. Body weight of high-dose females was 68% that of control body weight on the day of vaginal opening, and the effect was statistically significant. The study authors attributed the effect to DEHP-induced toxicity and not to an estrogenic effect. The study authors identified a lowest observed adverse effect level (LOAEL) of 375 mg/kg bw/day for this study based on a significant decrease in anterior prostate weight and increase in permanent nipple retention.

Li et al. *(87)* examined dose-related effects of DEHP on neonatal rat gonocytes and Sertoli cells. Male Sprague-Dawley rat pups from 4–7 litters were pooled and randomly placed into groups of 4 or 5 pups. On PND 3 (day of birth = PND 1), the rats were gavage dosed with DEHP at 0, 20, 100, 200, or 500 mg/kg bw. Pups were killed 24 hours after dosing and testes were collected for morphological examination and measurement of Sertoli cell proliferation through BrdU uptake. The time course of effects was examined in a second experiment where rats were dosed with 0 or 200 mg/kg bw DEHP and examined between 6 and 48 hours following exposure. In rats treated with 100–500 mg/kg bw DEHP, there was a dose-related increase in abnormally large gonocytes containing 2–4 nuclei. Multinucleated gonocytes were first detected at 12 hours following exposure to 200 mg/kg bw DEHP, and their numbers increased with time. Sertoli cell proliferation was reduced in rats treated with ≥100 mg/kg bw DEHP. Sertoli cell proliferation rebounded at 48 hours following treatment. DEHP did not affect serum FSH levels. **[Due to reporting deficiencies, only the Sertoli cell proliferation data were considered to be of utility.]**

Cammack et al. *(88)* examined reproductive development of Sprague-Dawley rats (n = 16/group) treated iv or orally with DEHP. Beginning at 3–5 days of age, rats were treated for 19–21 days with DEHP at 0, 60, 300, or 600 mg/kg bw/day by iv infusion or 0, 300, 600 mg/kg bw/day by oral gavage. Seven rats/group were scheduled to be killed following the dosing period and 9/group to be held for a recovery period until 90 days of age. Histopathological analyses of prostate, seminal, vesicle, and epididymis and an evaluation of sperm count, motility, and morphology were conducted in the 90-day-old rats. Body weight gain was decreased in rats given 600 mg/kg bw/day by iv infusion and oral gavage. In animals killed immediately after the dosing period, absolute testes weights were significantly reduced at iv and oral doses ≥300 mg/kg bw/day. Absolute liver weights were increased in rats given ≥300 mg/kg bw/day DEHP by iv infusion. Depletion of germinal epithelium and/or decreased seminiferous tubule diameter was noted in all animals from the 300 and 600 mg/kg bw/day oral and iv dosing groups. Germinal epithelium depletion was rated as moderate (51–75% reduction in thickness) in the 600 mg/kg bw/day oral group and mild (25–50% change) in all other groups given ≥300 mg/kg bw/day DEHP. Reduced tubule diameter was rated as mild (25–50% reduction in diameter) in the 600 mg/kg bw/day oral group and minimal (<25% change) in the other groups treated with ≥300 mg/kg bw/day DEHP. In animals killed at 90 days of age, reduced testicular weights persisted in iv and oral groups given ≥300 mg/kg bw/day. The only persisting testicular lesion was a minimal (<25%) decrease in seminiferous tubular diameter in 2 of 5 rats in the 300 mg/kg bw/day oral group and 3 of 7 rats in the 600 mg/kg bw/day oral group. No prostate, epididymis, or seminal vesicles lesions and no adverse effects on sperm count, motility, or morphology were observed.

A multigeneration DEHP toxicity study conducted in rats also provided some information on developmental toxicity *(113)*. The study is described in detail in Section 4. Briefly, offspring of rats that were fed DEHP in diet at 3000 and 9000 ppm (340 and 1088 mg/kg bw/day) during gestation and lactation experienced an increase in stillbirth, an increase in PND 0–4 pup mortality, retardation of F_2 pup body weight, altered male anogenital distance, and retained nipples/areolae. A delay in sexual maturation was also noted in F_1 offspring at the 9000 ppm exposure level.

Effects noted in numerous single dose-level studies were consistent to those observed in the multiple dose level studies summarized above. Although testicular weight and histology were not affected in offspring of rats treated with 100 mg/kg bw/day DEHP during pregnancy or lactation *(80)*, treatment of rats with DEHP 750 mg/kg bw/day in late pregnancy and/or early lactation resulted in decreased testicular weights and testicular lesions in offspring *(77, 79)*. Nipples and reduced anogenital distance were repeatedly observed in male offspring exposed to 750 mg/kg

bw/day DEHP during gestation or lactation *(77, 82, 84)*. Additional observations in male offspring of rats dosed with 750 mg/kg bw/day during late pregnancy and early lactation were lack of testicular descent, agenesis of accessory reproductive organs, and incomplete preputial separation *(77)*. In contrast to findings reported by Moore et al. *(81)*, a study by Gray et al. *(77)* using a similar design and dose level reported no apparent effects of DEHP exposure on sexual behavior, with the exception of an inability to achieve intromissions by males with malformed penises. Another inconsistency between the Gray et al. and Moore et al. findings was that Gray et al. reported no effect of DEHP treatment on sperm numbers **[data not shown]**.

A number of studies examined mechanisms of DEHP toxicity. Single dose-level studies with exposures during gestation and/or lactation and examination of fetal or immature rats consistently demonstrated reductions in blood testosterone levels at ≥100 mg/kg bw/day DEHP *(80, 82)*, Leydig cell testosterone production at ≥100 mg/kg bw/day *(80)*, testicular testosterone content at ≥300 mg/kg bw/day *(79, 82)*, and ex vivo testicular testosterone production at ≥750 mg/kg bw/day *(79, 82, 86)*. One of the studies indicated that reductions in testosterone production observed shortly after exposure in neonatal or weanling rats were no longer present in adulthood *(80)*.

Evidence that DEHP targets Leydig cells, gonocytes, and Sertoli cells was noted following gestational and lactational exposure of rats to ≥100 mg/kg bw/day DEHP *(79, 80, 87)*. MEHP, but not 2-ethylhexanol, was found to cause increases in large multinucleated gonocytes and to inhibit Sertoli cell proliferation *(87)*. An in vitro study demonstrated that gonocytes and Sertoli cells are susceptible to MEHP-induced toxicity during periods of proliferation *(92)*. In 1 study, DEHP doses ≥750 mg/kg bw/day during gestation in rats reduced testicular expression of insulin-like hormone 3, a hormone produced by Leydig cells and possibly involved in development of the gubernaculum *(86)*.

A study conducted in cells transfected with the human androgen receptor vector pCMVhAR found that neither DEHP nor MEHP competed for androgen receptor binding at concentrations up to 10 μM *(79)*.

Additional developmental toxicity studies examined possible effects of DEHP exposure on zinc metabolizing enzymes, pulmonary toxicity, and neurobehavior.

Lee et al. *(100)* examined the expression of zinc metabolizing enzymes in mouse dams and embryos exposed to DEHP. In the first part of the study, gavage administration of 800 mg DEHP/kg bw on GD 9 increased maternal liver expression of MT-I and MT-II, enzymes that sequester zinc in the liver, but had no effect on maternal liver expression of ZnT-1, a transmembrane protein involved in zinc efflux. In embryos MT-I and MT-II expression were down-regulated and ZnT-1 expression was up-regulated in brain. In a dose-response study, where the dams were given DEHP 50–800 mg/kg bw on GD 9, changes in expression of zinc metabolizing enzymes were first observed at 200 mg/kg bw/day in dams (MT-1 and MT-II) and 50 mg/kg bw/day in embryos (MT-II). This paper was considered useful only in hypothesis generation.

Tanaka *(95)* examined neurobehavioral toxicity in offspring of mice exposed to DEHP through diet during gestation and lactation. DEHP was added to diet at 0, 0.01, 0.03, or 0.09%. Doses during gestation were 0, 17, 47, and 140 mg/kg bw/day, and doses during lactation were 0, 60, 172, and 493 mg/kg bw/day. At birth (PND 0), litter size, litter weight, and sex ratio were determined. Body weight gain, survival, and neurobehavioral parameters (surface righting, negative geotaxis, cliff avoidance, swimming behavior, and olfactory orientation) were monitored

during the lactation period. One male and 1 female per litter were selected to continue receiving treatment until 9 weeks of age. Doses in offspring were 0, 16–19, 48–56, and 145–171 mg/kg bw/day. Additional neurotoxicity testing (water T-maze and exploratory behavior) was conducted at 7–8 weeks of age. In F_0 mice, DEHP treatment had no effect on body weight gain, movement, or exploratory activity. There were no significant effects on sex ratio or litter size or weight at birth. A 7% decrease in body weight in male offspring of the low-dose group compared to control males on PND 0 was the only significant body weight effect observed in offspring. Significant reductions in survival were noted in the high-dose group for female offspring from PND 4 to 14 and for total offspring from PND 4 to 21. Total surviving offspring at PND 21 was 98.4% in the control group and 92.8% in the high-dose group. Time for surface righting was significantly delayed in low- and mid-dose females on PND 4, in high-dose males on PND 7, and in low-dose females on PND 7. There were no other significant findings in neurobehavioral parameters examined during the lactation period. There were no adverse effects in water T-maze performance in treated animals at 7 weeks of age, and movement and exploratory behavior were not affected by treatment at 3 or 8 weeks of age. The study authors concluded that few adverse effects were observed at the DEHP doses used in this study. **[The Expert Panel noted concerns about the conduct and interpretation of the behavioral tests.]**

Table 25. Summary of DEHP Effects on Developmental Toxicity

Species and strain	Treatment	Effect levels/Benchmark doses [a]	Reference
Sprague-Dawley rat	Oral at 0, 375, 750, or 1500 mg/kg bw/day from GD 3 to PND 21.	Maternal LOAEL 750 mg/kg bw/day (reduced prenatal weight gain) **[BMD$_{10}$ 433 mg/kg bw/day; BMDL$_{10}$ 317 mg/kg bw/day; BMD$_{1\,SD}$ 754 mg/kg bw/day; BMDL$_{1\,SD}$ 509 mg/kg bw/day]**. Developmental LOAEL 375 mg/kg bw/day (increase in areolas or nipples, incomplete preputial separation, non-descent of testes, and agenesis of anterior prostate). **[Benchmark doses could not be calculated for LOAEL endpoints listed above. The lowest calculable BMD$_{10}$ values were obtained for epididymal sperm numbers: BMD$_{10}$ 213 mg/kg bw/day and BMDL$_{10}$ 167 mg/kg bw/day. The lowest BMD$_{1\,SD}$ values were for PND 105 epididymis weight: BMD$_{1\,SD}$ 220 mg/kg bw/day and BMDL$_{1\,SD}$ 135 mg/kg bw/day.]**	Moore et al. *(81)*
Sprague-Dawley rat	Gavage at 0, 20, 100, 200, or 500 mg/kg bw on PND 3 (day of birth = PND 1).	Developmental LOAEL 100 mg/kg bw/day (reduced Sertoli cell proliferation)	Li et al. *(87)*
Wistar rat	Feed at 0, 1000, 3000, and 9000 ppm (0, 113, 340, and 1088 mg/kg bw/day) during gestation and lactation.	Maternal LOAEL 1088 mg/kg bw/day (decreased feed intake and body weight gain) Developmental LOAEL 340 mg/kg bw/day (increased pre- and postnatal mortality, decreased body weight, altered male anogenital distance, and retained nipples/areolae). **[Lowest developmental benchmark dose was for F$_2$ pup survival on PND 0–4: BMD$_{10}$ 2325 ppm and BMDL$_{10}$ 2045 ppm (263 and 231 mg/kg bw/day, based on dose regression provided by the authors).]**	Schilling et al. *(113)*, discussed in Section 4.
Sprague-Dawley rat	Gavage at 0, 300, or 600 mg/kg bw/day for 19–21 days beginning at 3–5 days of age.	*Examination immediately after dosing:* Developmental LOAEL 300 mg/kg bw/day (depletion of germinal epithelium and/or decreased seminiferous tubule diameter and decreased absolute testes weight). **[Testes weight: BMD$_{10}$ 90.7 mg/kg bw/day, BMDL$_{10}$ 77.4 mg/kg bw/day, BMD$_{1\,SD}$ 875 mg/kg bw/day, BMDL$_{1\,SD}$ 628 mg/kg bw/day; liver weight: BMD$_{10}$ 712 mg/kg bw/day, BMDL$_{10}$ 196 mg/kg bw/day, BMD$_{1\,SD}$ 585 mg/kg bw/day, BMDL$_{1\,SD}$ 193 mg/kg bw/day.]** *Examination at 90 days of age:* Developmental LOAEL 300 mg/kg bw/day (decrease in seminiferous tubular diameter, decreased absolute testes weight).	Cammack et al. *(88)*

Species and strain	Treatment	Effect levels/Benchmark doses [a]	Reference
Sprague-Dawley rat	iv at 0, 60, 300, or 600 mg/kg bw/day for 21 days beginning at 3–5 days of age.	**[Testes weight: BMD_{10} 138 mg/kg bw/day, $BMDL_{10}$ 125 mg/kg bw/day, $BMD_{1\,SD}$ 85 mg/kg bw/day, $BMDL_{1\,SD}$ 65 mg/kg bw/day.]** *Examination immediately after dosing:* Developmental LOAEL 300 mg/kg bw/day (depletion of germinal epithelium and/or decreased seminiferous tubule diameter, decreased absolute testes weight, and increased absolute liver weight). **[Testes weight: BMD_{10} 122 mg/kg bw/day, $BMDL_{10}$ 106 mg/kg bw/day, $BMD_{1\,SD}$ 179 mg/kg bw/day, $BMDL_{1\,SD}$ 125 mg/kg bw/day; liver weight: BMD_{10} 163 mg/kg bw/day, $BMDL_{10}$ 122 mg/kg bw/day, $BMD_{1\,SD}$ 101 mg/kg bw/day, $BMDL_{1\,SD}$ 66 mg/kg bw/day.]** *Examination at 90 days of age:* Developmental LOAEL 300 mg/kg bw/day (decreased absolute testes weight) **[BMD_{10} of 222 mg/kg bw/day, $BMDL_{10}$ 190 mg/kg bw/day, $BMD_{1\,SD}$ 169 mg/kg bw/day, $BMDL_{1\,SD}$ 105 mg/kg bw/day.]**	Cammack et al. *(88)*
CD-1 mouse	Feed at 0, 17, 47, and 140 mg/kg bw/day during gestation, 0, 60, 172, and 493 mg/kg bw/day during lactation, and 0, 16–19, 48–56, and 145–171 mg/kg bw/day from weaning to 9 weeks of age in F_1 offspring.	Maternal NOAEL 140–493 mg/kg bw/day Developmental LOAEL 140–493 mg/kg bw/day (decreased survival during lactation period).	Tanaka *(95)*

[a]See the footnote to Table 20 for definitions and a discussion of the use of benchmark dose in this report.

Conclusions from the original Expert Panel evaluation

The original Expert Panel report on DEHP contained conclusions about developmental toxicity in Section 5. These conclusions have been extracted and reproduced below, with the section numbering as found in the original document. The references listed in the conclusion are listed, and the table to which the conclusions refer is reproduced, numbered Table 71 as in the original.

5.1.3
There were no studies located on the developmental toxicity of DEHP or its metabolites in humans.

5.1.3.1
Developmental toxicity findings were remarkably consistent. DEHP was found to produce malformations, as well as intrauterine death and developmental delay. The pattern of malformations seen in fetuses is consistent across studies. It included morphological abnormalities of the axial skeleton (including tail), cardiovascular system (heart and aortic arch), appendicular skeleton (missing limb bones, finger abnormalities), eye (including open eye), and neural tube (exencephaly).

In general, across studies there was not a strong relationship between the type and amount of maternal toxicity and developmental toxicity.

In addition to the studies of developmental toxicity with post-conception exposure discussed above, developmental toxicity was also manifested in reproductive toxicity studies . . .. The database as a whole identified CD-1 mice as the most sensitive species for DEHP developmental toxicity via the oral route. The critical papers are [149,150,168,186]. LOAELs and NOAELs for some relevant developmental toxicity studies are presented in Table 71. Developmental effects in reproduction studies are listed in Table 76 **[See Section 4 of this report.]** Studies that address developmental toxicity are consistent in identifying the lower effective range of oral exposure, taking into account differences in duration of treatment.

The DEHP database contains four rat studies conducted by a route other than oral: an IV study, two IP studies, and an inhalation study. These studies provide valuable information but do not contain enough data for separate route-specific hazard identification and NOAEL/LOAEL selection.

The panel is not confident that the lowest dose has been established at which developmental toxicity (the development of the male reproductive system) occurs.

5.1.3.6
The database provides adequate information to identify DEHP as a developmental toxicant by the oral route and for identification of NOAELs and LOAELs for dose–response assessment. The data are also sufficient to identify the metabolites (MEHP, 2-EH, 2-EHA) as developmental toxicants. However, there are not enough studies for independent hazard identification and dose–response assessment for the parenteral route. Because of the known role of intestinal lipase in DEHP metabolism, it is not possible to readily generalize dose–response assessment from the oral to intravenous route. Existing PBPK models do not include fetal compartments; and hence have limited use at present.

5.2.

As will be discussed below, there are sufficient data in rodents to conclude confidently that oral exposure to DEHP can cause reproductive and developmental toxicity in rats and mice. Further, an effect observed in rats involves adverse effects on the development, structure, and function of the male reproductive tract. Thus, for DEHP, the effects on reproduction and development are intertwined.

The developmental toxicity database contains well-conducted and reported studies, many available as full GLP study reports, and additional, more restricted studies that provide supplemental and supportive information. The database is somewhat limited in that it consists almost entirely of studies in rats and mice orally exposed during gestation where effects are seen by examining physical development of rodent pups just prior to birth (i.e. prenatal assessment). These studies indicate that a range of effects may occur, including malformations (tail malformations, axial and appendicular skeletal abnormalities, cardiovascular malformations, and neural tube closure defects), developmental delays, and intrauterine death. The NOAEL based on malformations in rodents was ~40 mg/kg bw/day and a NOAEL of 3.7–14 mg/kg bw/day was identified for testicular development/effects in rodents. In contrast, functional reproductive endpoints that are evaluated through postnatal observation have not been adequately studied. This is a significant data limitation. There are a limited number of studies by the inhalation, dermal, and intravenous administration routes. It was noted that results are consistent across studies, taking into account doses, route, species, timing,

The examination of effects during the late gestational and neonatal periods is quite recent and incomplete. Despite the general belief among expert panel members that this represents a time of potentially high sensitivity to DEHP-induced disruption of the reproductive system, the dose–response relationships for reproductive effects following exposures in gestational versus postnatal ages are unknown. Low-dose studies examining sensitive endpoints following late gestational exposure are a critical data need.

There is a study that demonstrated the same spectrum of developmental toxicity (as seen in 'normal' mice) in mice that were genetically incapable of expressing peroxisome proliferation due to lack of PPAR-alpha.

DEHP data from rats and mice are assumed relevant to judging hazard to human reproduction and development; they are the standard mammalian test systems used.

References from the original report cited above

[146] Price CJ, Tyl RW, Marr MC, Myers CB, Sadler BM, Kimmel CA. Reproduction and fertility evaluation of diethylhexyl phthalate (CAS No. 117-81-7) in CD-1 mice exposed during gestation. Research Triangle Park, NC: National Toxicology Program, 1988.

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[186] Reel JR, Tyl RW, Lawton AD, Jamb JC. Diethylhexyl phthalate (DEHP): Reproduction and fertility assessment in CD-1 mice when administered in the feed. PB84-181734. Springfield, VA: available from: NTIS, Research Triangle Park: National Toxicology Program,1984.

Table 71. Summary of DEHP effects in developmental toxicity studies with oral exposure.				
Protocol & Doses	NOAEL (mg/kg bw/day)	Maternal LOAEL (mg/kg bw/day)	Developmental LOAEL (mg/kg bw/day)	Fetal Effects at Higher Doses
Prenatal feeding study in CD-1 mice. 30/group received 0, 44, 91, 191, or 293 mg/kg bw/day on gd 0-17. Dams and pups examined in late gestation. [149, 185][a].	44 for maternal 44 for developmental	91 Clinical signs.	91 ↑ Skeletal, visceral, and external malformations.	↑ Skeletal, visceral, and external malformations. ↑ Prenatal mortality. ↓ Fetal weight.
Prenatal gavage study in CD-1 mice. 14 /group received 0, 40, 200, or 1000 mg/kg bw/day on gd 6-15. Dams and pups examined in late gestation. [150][a].	200 for maternal. 40 for developmental.	1000 ↑ Liver weight. ↓ Weight gain.	200 ↑ Visceral and external variations and malformations.	↑ Skeletal, visceral, and external variations and malformations. ↑ Prenatal mortality.
Prenatal feeding study in Fischer 344 rats. 20/group received 0, 164, 313, or 573 mg/kg bw/day on gd 0-20. Pups evaluated postnatally. [156][a]	164 for maternal 164 for developmental	313 ↓ Food Intake.	313 ↑ Prenatal mortality.	↑ Prenatal mortality. ↓ Pup body weight on pnd 1 only.
Prenatal feeding study in CD-1 mice. 28/group received 0, 19, 48, or 95 mg/kg bw/day from gd 0-17. [146][a]	95 for maternal 48 for developmental	No higher doses.	95 ↑ Prenatal mortality. ↓ Pup survival on pnd 4.	No higher doses.
[a]Doses calculated by study author.				

Questions for the Expert Panel

Are human data sufficient for an evaluation of the developmental toxicity of DEHP following prenatal exposure?

If so, what are the relevant exposure conditions and endpoints?

Are human data sufficient for an evaluation of developmental toxicity of DEHP following exposure of children?

If so, what are the relevant exposure conditions and endpoints?

How, if at all, are the conclusions about the human developmental effects of DEHP different from those in the first Expert Panel report?

Are experimental animal data sufficient for an evaluation of the developmental toxicity of DEHP following prenatal or lactational exposure or other exposures of immature animals?

If so, what are the relevant experimental animal models, exposure conditions, and endpoints?

If the experimental animal data are sufficient for an evaluation, are the data assumed relevant, relevant, or not relevant?[a]

How, if at all, are the conclusions about the experimental animal developmental effects of DEHP different from those in the first Expert Panel report?

[a]The definitions of the term sufficient and the terms assumed relevant, relevant, and not relevant are in the CERHR guidelines at http://cerhr.niehs.nih.gov/news/guidelines.html.

4.0 REPRODUCTIVE TOXICITY DATA

4.1 Human Data

Modigh et al. *(114*), supported by the Swedish Environmental Protection Agency and Swedish Work Environment Fund, evaluated time to pregnancy in the partners of men potentially exposed to DEHP. Men employed or the partners of women employed in 1 of 3 plants were invited to participate. Among the 284 men identified as eligible, 234 responded. The responders had produced 397 pregnancies. After excluding pregnancies for which information was unavailable, 326 pregnancies were available for analysis. Information on time to pregnancy was obtained in a telephone or written interview in which couples were asked how many months they had unprotected intercourse prior to achieving pregnancy. Pregnancies were counted if the couple was attempting to become pregnant or not attempting but not avoiding pregnancy. Only pregnancies ending in 1987 or later were counted. Information was accepted from either partner; the woman's answer was used if there was disagreement. Exposure was estimated from employed subjects' description of work tasks and measurements that had been made in each of the 3 workplaces during the general time period relevant for the pregnancies. Exposure categories were created as follows: *unexposed* pregnancies (n = 182) were fathered by operators who were not exposed during the time leading up to pregnancy, by office staff, or unexposed male partners of female workers; *low-exposure* pregnancies (n = 100) were fathered by men with estimated non-zero DEHP exposures < 0.1 mg/m^3; and *high-exposure* pregnancies (n = 44) included 25 pregnancies fathered by men exposed to DEHP 0.1–<0.2 mg/m^3, 15 pregnancies fathered by men exposed to DEHP 0.2–<0.5 mg/m^3, and 4 pregnancies fathered by men with DEHP exposures of 0.5 mg/m^3 or higher. The highest estimated mean DEHP exposure level was 1.9 mg/m^3. To account for possible effects at any time during the 70-day period of spermatogenesis, exposures were evaluated 1, 2, and 3 months prior to the month of attempted pregnancy. Fecundability ratios calculated from a Cox proportional hazards model were estimated using the unexposed pregnancies as the referent. A binomial regression model was used to control for potential confounders (father's age, mother's age, and length of time to recall). An additional analysis was conducted using only the first pregnancy from couples for which more than 1 pregnancy was available.

Median time to pregnancy was 3.0 months in the unexposed group, 2.25 months in the low-exposure group, and 2.0 months in the high-exposure group. The crude and adjusted fecundability ratios for the exposed pregnancies were all close to 1.0, and the 95% confidence intervals all overlapped unity. There was no significant effect of restricting the analysis to the first pregnancy of couples with more than 1 pregnancy or of excluding pregnancies conceived by employed women. Excluding couples with known fertility problems did not influence the findings. The results did not depend on whether exposure status was used for the month under consideration or lagged 1, 2, or 3 months. The authors concluded that there was no evidence of a DEHP-associated prolongation in time to pregnancy, although they recognized that there were few highly exposed men in their sample; the mean DEHP exposure level for men in the study was less than 0.5 mg/m^3.

Strengths/Weaknesses: Time to pregnancy can be a sensitive marker of reproductive impairment. The use of unexposed men from the same workplaces as referents is a strength. Measurements of exposure were objective and independent of self-reports of work tasks and locations. The study considered numerous potential confounders. Weaknesses include the small number of highly exposed men and measurement error involved in the retrospective assessment of paternal exposure. The use of only men who fathered pregnancies is a limitation of the

retrospective time to pregnancy assessment. Other weaknesses include the low response rates and the inability to mask participants to exposure and outcome status.

Utility (Adequacy) for CERHR Evaluation Process: This paper is very useful in the evaluation process.

Rozati et al. (115), support not indicated, measured phthalate esters in the seminal plasma of 21 men with unexplained infertility. The men were male partners in couples presenting for infertility evaluation **[not otherwise defined]**. All subjects had a sperm concentration <20 million/mL, rapidly progressive motility <25%, total progressive motility <50%, or <30% normal forms. Sperm concentration was assessed using a hemocytometer, and morphology was assessed after Papanicolaou staining. **[Details on motility evaluation were not provided. The number of semen samples per subject was not indicated.]** Additional testing included eosin-nigrosin staining to determine vitality, hypo-osmotic swelling test, chromatin decondensation after treatment with SDS and EDTA, and chromatin susceptibility to acid denaturation, determined with acridine orange staining and fluorescent microscopy. Seminal fluid concentrations of phthalate esters **[as a group]** were assessed by HPLC, using a commercial phthalate esters mixture as a standard. **[According to the manufacturer's web site, this mixture contains 0.2% each DEHP, di-*n*-octyl, dimethyl, diethyl, di-*n*-butyl, and benzyl butyl phthalate in hexane (http://www.sigmaaldrich.com/cgi-bin/hsrun/Suite7/Suite/HAHTpage/Suite.HsSigma AdvancedSearch.formAction, accessed April 27, 2005).]** Comparison was made to seminal plasma phthalate concentrations in a control group of 32 men with evidence of conception and normal semen analysis **[not otherwise characterized]** using Student t test. Correlation between seminal phthalate ester concentration and individual sperm test results was evaluated using linear regression analysis. **[Regression terms were not specified. Polychlorinated biphenyl concentration was also evaluated in seminal plasma. It is not stated whether the regression analysis adjusted for polychlorinated biphenyl concentration.]**

The mean ± SD seminal plasma phthalate ester concentration in the infertile group was 2.03 ± 0.214 µg/mL, compared to 0.06 ± 0.02 µg/mL in the control group ($P < 0.05$). There was a significant inverse correlation between seminal phthalate ester concentration and normal sperm morphology ($r = -0.769$, $P < 0.001$) and a positive correlation between seminal phthalate ester concentration and the percent acid-denaturable sperm chromatin ($r = 0.855$, $P < 0.001$). There was no significant correlation between seminal phthalate ester concentration and ejaculate volume, sperm concentration, progressive motility, sperm vitality, sperm osmoregulation, or sperm nuclear chromatin decondensation.

The authors concluded that adverse effects on fertility of phthalate esters, which they called xenoestrogens, were consistent with published data on male reproductive toxicity of these compounds.

Strengths/Weaknesses: There was extensive reproductive assessment of cases, but the sample size was small, and there was very little information on the selection of controls for infertile cases. There was very limited assessment of possible confounders (mean age, urban/rural, fish consumption) and no evidence that exposure assessment was carried out blind to case/control status of participants.

Utility (Adequacy) for CERHR Evaluation Process: This report is somewhat useful in the evaluation process.

Duty et al. *(116*), supported by NIEHS, evaluated urinary MEHP and semen analysis parameters. Subjects included 168 men being evaluated in a clinic as part of a fertility evaluation. A questionnaire was used to obtain information on lifestyle factors. A single semen sample was produced by masturbation after instructions to abstain from ejaculation for 48 hours. Sperm concentration and percent motility were assessed using computer-assisted sperm analysis, and morphology was evaluated by light microscopy of air-dried smears after application of a commercial stain (Diff-Quik). A single spot urine was collected on the same day as the semen sample and analyzed using HPLC with tandem MS for MEHP and for monoethyl, monomethyl, mono-*n*-butyl, monobenzyl, mono-*n*-octyl, mono-isononyl, and monocyclohexyl phthalates. Urine phthalate concentrations were adjusted based on urine specific gravity and were dichotomized as high or low based on median values. Sperm parameters were dichotomized based on published norms. Abnormal sperm concentration was <20 million/mL, abnormal motility was <50% motile, and abnormal morphology was <4% normal forms. Mantel-Haenszel chi-squared test was used to assess the relationship between high/low phthalate concentration in the urine and normal/abnormal semen parameter. Multivariate logistic regression was used to adjust for smoking status, age, race, body mass index, and abstinence time.

The study population included 28 men (17%) with low sperm concentration, 74 men (44%) with <50% motility, and 77 men (46%) with >4% normal forms. There were 77 men (46%) who were normal in all three domains. There were no significant associations between abnormal semen parameters and MEHP urine concentration above or below the group median. **[Associations were identified and explored with respect to monobutyl, monobenzyl, and monomethyl phthalate, not discussed here.]** The authors did not present conclusions relative to MEHP. In this paper and in the following papers from this group, the authors discussed limitations of their methods:
  ▪ Use of single spot urines. The authors indicated that because phthalates are rapidly eliminated, use of a single spot urine assumes steady state exposure from food and personal care products.
  ▪ Adjustment of urine phthalate concentrations using specific gravity. The authors acknowledge that creatinine is often used to adjust urine concentrations, but argue that use of creatinine may not be appropriate for compounds that are not excreted through glomerular filtration. They further note the dependence of creatinine secretion on muscle mass, physical activity, time of day, diet, urine flow, and disease states.
  ▪ Use of a fertility population. The authors expressed doubt that a fertility sample would include men that necessarily differ from men in the general population in their testicular response to phthalate exposure.

Strengths/Weaknesses: The detection of phthalate metabolites in urine eliminated contamination issues. There was good evaluation of confounders, but the use of a subfertile population is a weakness.

Utility (Adequacy) for CERHR Evaluation Process: This study is very useful in the evaluation process.

Duty et al. *(19*), supported by NIEHS, evaluated urinary MEHP and sperm motion parameters by computer-assisted sperm analysis. Subjects were the male partners in couples presenting for fertility evaluation, without regard to whether the male has a fertility problem. Of the 259 men who agreed to participate, 234 provided a urine sample and a semen sample. **[It is assumed, though not stated, that some of these subjects were also reported in Duty et al. *(116*).]** Thirteen semen samples did not contain motile sperm and 1 semen sample was not submitted for computer-assisted analysis, leaving 220 subjects with motile sperm measurements and spot urine samples. After elimination of urine samples with specific gravities below 1.010 or above 1.030,

187 semen-urine pairs remained for evaluation. Urine samples were frozen for subsequent phthalate monoester analysis by HPLC and tandem MS. The phthalate monoesters included the monoethyl, monomethyl, mono-*n*-butyl, monobenzyl, mono-*n*-octyl, mono-isononyl, and monocyclohexyl, as well as MEHP. Urinary phthalate determinations were normalized using specific gravity. Multiple linear regression analysis was used to evaluate the association between tertile of normalized urinary MEHP concentration and sperm motion parameter. Covariates included in the model were smoking status, race, age, body mass index, and abstinence interval prior to collection of semen.

Subjects had a mean ± SD age of 36.3 ± 5.6 years. Mean ± SD sperm concentration was 115.6 ± 99.2 million/mL with 13.2% of samples having a sperm concentration <20 million/mL. Mean ± SD percent sperm motility was 52.2 ± 22.6% with 41.8% having <50% motile sperm. Mean ± SD percent normal morphology was 7.4 ± 4.6% with 22.3% having <4% normal forms. **[The cut-offs representing the norms cited in Duty et al. (116).]** The authors stated that there was evidence of a dose–response relationship with respect to MEHP and straight-line velocity, curvilinear velocity, and linearity with *P* values for trends of 0.1–0.3. They further stated that use of quartiles instead of tertiles, use of phthalate concentration as a continuous parameter, and use of actual phthalate concentrations rather than concentrations adjusted for specific gravity produced results that were consistent with the initial analysis. **[Data and analyses were not shown.]**

The authors concluded that there was an overall pattern of decline in straight-line velocity, curvilinear velocity, and linearity, which was identified with mono-*n*-butyl and monobenzyl phthalate as well as MEHP. They postulated that the lack of statistical significance may have reflected the relatively small sample size. They indicated that if phthalates were associated with sperm motion abnormalities, their study may have under-ascertained the effect because immotile sperm did not give rise to motility parameters.

Strengths/Weaknesses: The detection of phthalate metabolites in urine eliminated contamination issues. There was good evaluation of confounders, but the use of a subfertile population is a weakness. This report appears to include the same subjects as Duty et al. *(116)* and cannot be considered independent.

Utility (Adequacy) for CERHR Evaluation Process: This study is very useful in the evaluation process.

Duty et al. *(117)*, supported by NIEHS, evaluated a possible association between urinary phthalate monoester concentrations and sperm DNA damage, assessed using the neutral comet assay **[so named because fragmented DNA streams away from the main cell body on electrophoresis, producing a visual image that looks like a comet]**. The subjects and samples were as described in Duty et al. *(19)*, although the number of samples tested was lower (n = 141). Semen samples were frozen prior to comet assay. Urinary phthalate monoester concentrations were adjusted for specific gravity and analyzed in quartiles using multiple linear regression adjusted for smoking status, race, age, body mass index, and abstinence interval prior to collection of semen. There were no significant associations between comet assay parameters and MEHP urinary concentrations. **[Significant associations were identified only for monoethyl phthalate.]** Inclusion of urine samples that had been excluded based on specific gravities below 1.010 or above 1.030 did not change the results. The authors did not express conclusions relative to MEHP.

Strengths/Weaknesses: The detection of phthalate metabolites in urine eliminated contamination issues. There was good evaluation of confounders, but the use of a subfertile population is a

weakness. This report appears to include the same subjects as Duty et al. *(116*) and cannot be considered independent.

Utility (Adequacy) for CERHR Evaluation Process: This study is very useful in the evaluation process.

Duty et al. (118), supported by NIEHS, evaluated the relationship between serum concentrations of testosterone, sex hormone-binding globulin, inhibin B, FSH, and LH and phthalate monoester concentrations in spot urine samples. The subjects included 295 men attending a clinic as part of a fertility evaluation. **[It is not known how many of these men were also included in the previously discussed studies from this group *(19, 116, 117*).]** Blood, semen, and urine samples were collected. Serum was frozen until assayed using RIA (testosterone), enzyme immunometric assay (sex hormone-binding globulin), enzyme-linked immunosorbent assay (inhibin B), or microparticle enzyme immunoassay (LH, FSH). Urine concentrations of MEHP and monomethyl, monoethyl, mono-*n*-butyl, and monobenzyl phthalate were assayed using HPLV with tandem MS and were adjusted based on urine specific gravity. Spearman correlation coefficients were calculated in the exploratory analysis followed by multiple linear regression with adjustment for smoking status, age, race, body mass index, previous fertility evaluation, prior fathering of a pregnancy, season, and time of day.

In their primary analysis, using all urine samples, the authors identified a "negative non-significant association" between urine MEHP concentration and serum testosterone, with a change in serum testosterone of −0.47 nmol/mL (95% CI −1.03 to 0.10, $P = 0.10$) for each quartile increase in MEHP concentration. In a secondary analysis, in which urine samples were excluded if they had a specific gravity <1.010 and >1.030, the association between MEHP concentration and testosterone was described as weaker (−0.42 ng/mL for each quartile increase; 95% CI −1.05 to 0.21; $P = 0.19$). **[Additional associations were identified between urinary mono-*n*-butyl and monobenzyl phthalate and serum concentrations of inhibin B and FSH.]**

The authors did not draw conclusions with respect to MEHP. They indicated that they could not tell whether the associations they identified represented physiologically relevant changes or were the result of conducting multiple comparisons.

Strengths/Weaknesses: The detection of phthalate metabolites in urine eliminated contamination issues. There was good evaluation of confounders, but the use of a subfertile population is a weakness. This report appears to include the same subjects as Duty et al. *(116*) and cannot be considered independent.

Utility (Adequacy) for CERHR Evaluation Process: This study is very useful in the evaluation process.

Cobellis et al. (119), support not indicated, measured DEHP and MEHP concentrations in the plasma and peritoneal fluid of 35 women identified by laparoscopy as having endometriosis. The operations were performed for ovarian cysts, chronic pelvic pain, or dysmenorrhea. A comparison group consisted of 24 age-matched controls without known reproductive disease **[laparoscopy was not performed on these women, and it is not known how many of them may also have had endometriosis]**. Blood samples were collected from the women undergoing surgery either the day prior to the procedure or "immediately before anaesthesia for laparoscopy." **[The paper does not indicate how many, if any, of the patients were receiving iv infusions at the time of sampling. The Expert Panel notes that Section 1.2.4 of the original CERHR report contains information on the amount of DEHP that can be transferred by medical infusions.]** Blood

samples were obtained from the age-matched controls at the same phases of the menstrual cycle as the surgical patients. Estimation of DEHP and MEHP concentration was by HPLC. The proportions of women in each group with detectable concentrations were compared using the Fisher exact test, and concentrations were compared using the Wilcoxon test. Correlation between stage of endometriosis (a semi-quantitative estimate of the extent of visible endometriosis implants) and DEHP/MEHP concentration was made by Spearman correlation coefficient. There was no difference in the proportion of women in either group with detectable DEHP or MEHP (91.4% of surgical patients compared to 92.6% of control women). The median concentration (interquartile range) of DEHP in the patients was 0.57 (0.06–1.23) µg/mL compared to a control value of 0.18 (0–0.44) g/mL ($P = 0.0047$). The median concentration (interquartile range) of MEHP in the patients was 0.38 (0.1–0.97) µg/mL compared to a control value of 0.58 (0.34–0.71) g/mL ($P = 0.12$). There was no significant association between DEHP/MEHP concentration in plasma or peritoneal fluid and stage of endometriosis in women undergoing laparoscopy. The authors concluded that there could be a plausible causal relationship between DEHP exposure and endometriosis, but that, "further studies are needed in order to elucidate the mechanisms underlying the observed statistical association."

Strengths/Weaknesses: Weaknesses include possible exposure of cases due to medical procedures, very limited information on the selection of controls for endometriosis cases, very limited evaluation of confounding, and small sample size

Utility (Adequacy) for CERHR Evaluation Process: This study is of limited utility in the evaluation process.

4.2 Experimental Animal Data

4.2.1 Female

Lovekamp and Davis (120), supported by NIEHS, evaluated the effects of MEHP on F344 rat granulosa cells in culture. Four-week-old animals were stimulated with diethylstilbestrol or with pregnant mare serum gonadotropin (PMSG) following which ovaries were removed and granulosa cells harvested. Cells were cultured in the presence of 500 nM testosterone and 200 ng/mL FSH. MEHP **[purity not specified]** was added to cultures at concentrations of 0–200 µM **[0–55.6 mg/L]** in a comparison with other phthalate monoesters and with Wy-14,643, another peroxisome proliferator. After 48 hours, estradiol and progesterone were measured in media using commercial RIA kits. Ribonucleic acid (RNA) was extracted from cells and RT-PCR performed to quantify messenger RNA (mRNA) for aromatase and for cholesterol side-chain cleavage enzyme. Aromatase protein was quantified by Western blot. Statistical comparisons were made using the Student *t* test or ANOVA, followed by least significant difference test.

A comparison with other phthalate monoesters showed a decrease in estradiol in the medium (controlled for protein content) with MEHP 100 or 200 µM **[27.8 or 55.6 mg/L]** but not with comparable molar concentrations of monomethyl, -ethyl, -propyl, -butyl, -pentyl, or-hexyl phthalate. Monopentyl phthalate was associated with a decrease in estradiol production at 400 µM. mRNA for aromatase was estimated in media after culture with MEHP 0, 25, 50, or 100 µM **[0, 7.0, 13.9, or 27.8 mg/L]**. Graphically, there appeared to be a concentration-dependent decrease in estradiol concentration and in aromatase mRNA; pair-wise comparisons with control were statistically significant for the 100 µM concentration for estradiol and for the 50 and 100 µM concentrations for aromatase mRNA. The peroxisome proliferator Wy-14,643 also decreased estradiol and aromatase mRNA. Cholesterol side-chain cleavage enzyme was not altered by MEHP, suggesting specificity of the effect on aromatase mRNA. Aromatase protein was decreased by MEHP at concentrations of 100 and 200 µM **[27.8 and 55.6 mg/L]**. In a final

experiment, granulosa cells were incubated for 48 hours with MEHP 0 or 200 μM **[0 or 56 mg/L]** and 8-bromo-cyclic adenosine monophosphate (8-Br-cAMP) added for the last 24 hours. mRNA for aromatase and cholesterol side-chain cleavage enzyme and medium progesterone levels were increased by 8-Br-cAMP in the absence of MEHP. In the presence of MEHP, mRNA for aromatase and medium estradiol levels were suppressed, but there was no suppression of P450 side-chain cleavage enzyme mRNA or progesterone. The authors interpreted their results as consistent with transcriptional suppression of aromatase by MEHP independent of the FSH-cAMP pathway. They proposed a peroxisome proliferator-activated receptor (PPAR) pathway as a candidate mechanism for MEHP suppression of granulosa cell steroidogenic function.

Strengths/Weaknesses: The strengths of this study include appropriate technical proficiency and appropriate study controls and statistics, as well as examination of a set of structurally diverse phthalates. The Panel has confidence in the veracity of these results. This study identified a probable main point of interference with steroid production, showed that it was most affected by MEHP and not other phthalates (thus identifying MEHP as the main concern), and proposed a possible mechanism by which this interference might occur (PPAR mediation). This in vitro study comes after several in vivo studies that demonstrated low-estradiol-related changes in female rats, so relevance is another strength. Weakness includes examining cells from only 1 species and using MEHP of uncertain purity.

Utility (Adequacy) for CERHR Evaluation Process: The data point towards a sub-cellular site of action. Their utility in risk assessment would come in helping to identify vulnerable species as those having these target enzymes. The in vitro data are presumed relevant for in vivo protection when circulating blood levels of MEHP (or even better, tissue levels) are known.

Lovekamp-Swan et al. (121), supported by NIEHS, evaluated the interaction of MEHP with PPAR pathways as a mechanism for modifying ovarian steroidogenic functions. Granulosa cells were harvested from 4-week-old Fisher rats 24 hours after injection of PMSG. Cells were cultured for 48 hours with 500 nM testosterone and 200 ng/mL FSH with or without MEHP **[purity not specified]** 50 μM **[13.9 mg/L]**. Culture media were assayed for estradiol using a commercial RIA kit. RNA was extracted from cells and specific RT-PCR probes used to amplify mRNA for quantification. The mRNAs were chosen to reflect the activity of genes involved in the PPAR pathways, steroidogenesis, or phthalate toxicity. The mRNAs included aromatase, 17β-hydroxysteroid dehydrogenase IV (which metabolizes estradiol to estrone), cholesterol side-chain cleavage enzyme, the aryl hydrocarbon (Ah) receptor, cytochrome P450 1B1 (CYP1B1), epoxide hydrolase, and heart-fatty acid binding protein (H-FABP, which is associated with luteal transformation of granulosa cells). mRNA results were normalized to glyceraldehyde 3-phosphate dehydrogenase as an internal control.

To test the hypothesis that MEHP inhibition of granulosa cell aromatase is mediated through different PPAR isoforms, the experiments included the coadministration with MEHP of troglitazone (a PPARγ ligand), selective PPARα and γ agonists, a selective PPARγ antagonist, a selective retinoic acid X receptor (RXR) agonist (because a PPARγ:RXR heterodimer is believed to decrease aromatase), 9-cis-retinoic acid (which binds both RXR and the retinoic acid receptor, RAR), and 15-deoxy-$\Delta^{12,14}$-prostaglandin J$_2$ (a PPAR activator). Statistical comparisons were made using the Student t test or ANOVA, followed by the least significant difference test.

MEHP in culture reduced aromatase mRNA by >40% compared to control. The addition of a PPARγ antagonist partially reversed the decrease in aromatase mRNA. PPARα and γ agonists reduced aromatase mRNA to an extent similar to that after treatment with MEHP. To test the hypothesis that MEHP-activated PPARγ plus activated RXR results in aromatase suppression

mediated by the PPARγ:RXR heterodimer, cells were treated with MEHP plus RXR or RAR ligands. All treatments decreased aromatase, with significant additional suppression by MEHP when it was added with 9-*cis*-retinoic acid. To demonstrate that MEHP activity could be mediated through PPARα, MEHP treatment was shown to increase mRNA for 17β-hydroxysteroid dehydrogenase IV, which is inducible by PPARα. The addition of a PPARγ antagonist did not alter the MEHP induction of 17β-hydroxysteroid dehydrogenase IV, suggesting MEHP activation of the α-isoform of PPAR. Both MEHP and a selective PPARα agonist, but not a selective PPARγ agonist, increased the expression of Ah receptor, CYP1B1, and epoxide hydrolase. Cholesterol side-chain cleavage enzyme was not induced by MEHP or by either PPAR isoform agonist. H-FABP was induced by MEHP and by each specific PPAR isoform agonist. The authors concluded that MEHP effects on granulosa cell gene expression, which would serve to decrease estrogen production, were mediated by both PPAR pathways.

Strengths/Weaknesses: These data further dissect the molecular pathways by which MEHP reduces estradiol production in granulosa cells. The experiments were well-conceived and executed, used appropriate statistics, and the Expert Panel has confidence in the veracity of these studies. Possible weaknesses for the evaluation process include limited species evaluation, and relying on the presumption that these in vitro data report processes that are relevant and active in vivo.

Utility (Adequacy) for CERHR Evaluation Process: These in vitro data are adequate for the evaluation process and are presumed relevant for in vivo biology. An additional assumption implicit in these experiments is that immature stimulated rodent granulosa cells are appropriate models for human granulosa cells, and that the agonists and antagonists employed are specific for their intended targets. The data are uniquely valuable in describing a mechanism of toxicity that may be tested in other species, and that is presumed relevant for other species sharing a similar biochemistry.

Anas et al. *(122)*, supported by the Japanese Ministry of Agriculture, Forestry, and Fisheries, evaluated the effect of MEHP on in vitro maturation of bovine oocytes. Cumulus-oocyte complexes were obtained by aspirating 2–5 mm follicles from slaughterhouse beef ovaries. Oocytes with unexpanded cumulus layers were selected for study. In experiment 1 (n=91–99 oocytes/group), cumulus–oocyte complexes were cultured for 24 hours with MEHP (90% purity) at 0, 10, 25, 50, 75, or 100 μM [**0, 2.8, 7.0, 13.9, 20.9, or 27.8 mg/L**], after which they were evaluated for cumulus expansion and for stage of oocyte maturation. **[Maturation was assessed in ethanol-fixed orcein-stained oocytes using unspecified criteria.]** In experiment 2 (n=123–131 oocytes/group), denuded oocytes were cultured with MEHP at the same concentrations with the addition of a 5 μM **[1.4 mg/L]** concentration level, followed by assessment of oocyte maturation stage. In experiment 3, cumulus-oocyte complexes were cultured for 24 hours with MEHP 0, 50, or 100 μM **[0, 13.9, or 27.8 mg/L]**, following which some oocytes were evaluated for maturational stage. Other MEHP-treated oocyte cultures were continued in MEHP-free culture medium for an additional 24 hours or were continued at their original MEHP concentration (50 or 100 μM) for an additional 24 hours. The treatment groups (n=124–135 oocytes/group) consisted of MEHP exposure for 24 hours, MEHP exposure for 24 hours followed by 24-hour "recovery," and MEHP exposure for 48 hours. Statistical analysis was by ANOVA and Fisher protected least significance test. Experiments 1 and 2 showed a concentration-dependent decrease in progression through oocyte maturation stages, with a significant decrease in oocytes reaching metaphase-II beginning at MEHP 25 μM **[7.0 mg/L]** for cumulus-oocyte complex culture and at 10 μM **[2.8 mg/mL]** for denuded oocytes (Figure 2). Cumulus expansion was not impaired in experiment 1 **[suggesting no effect of MEHP on granulosa cell division**

and differentiation]. In experiment 3, impairment of progression to metaphase II was seen with the initial 24-hour culture with MEHP, as expected. Culture in MEHP-free medium permitted the progression of oocyte maturation to metaphase II in 64.5–71.1% of oocytes, although the proportion of oocytes reaching metaphase II did not recover completely to control levels (83.2% at 24 hours). The recovery of maturation ability suggested that the MEHP-associated decrease in oocytes reaching metaphase II was not likely to be due to nonspecific cytotoxic or lethal effects. When oocytes were cultured in MEHP 50 μM for 48 hours, the proportion reaching metaphase II was higher at 48 hours (41.6%) than at 24 hours (26.9%), suggesting that MEHP delayed maturation rather than preventing maturation altogether.
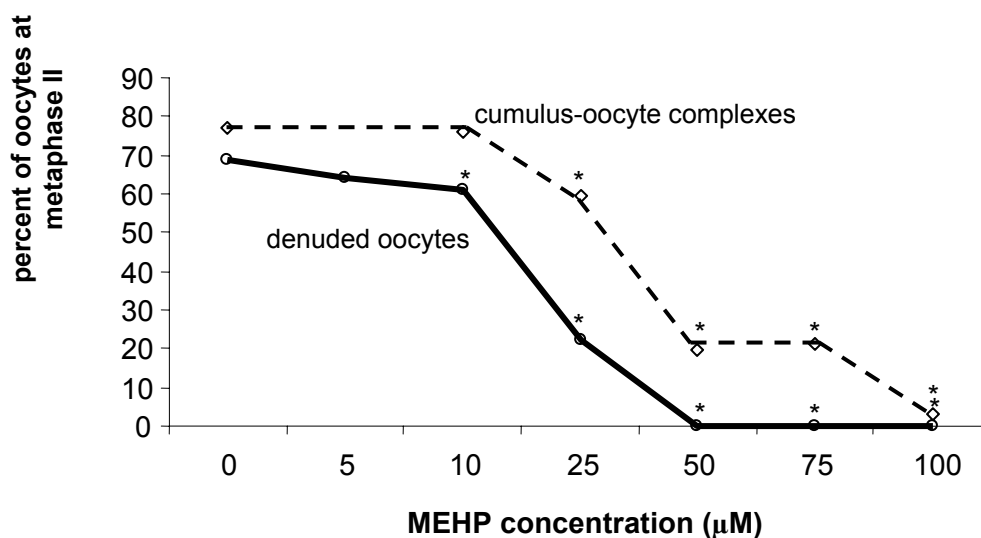
Figure 2. Percent of Bovine Oocytes Reaching Metaphase II after 24-hour Culture with MEHP

*Significant difference from 0 ppm group. Drawn from Anas et al. *(122)*.

Strengths/Weaknesses: The strengths of this study include very large sample sizes, reasonable statistics, a useful study design that included recovery, and a novel approach. It would have been more valuable to have included additional measures of cytotoxicity and perhaps to have gone past simple description to the use of specific agonists or antagonists (see Lovekamp et al. *(121)*, above).

Utility (Adequacy) for CERHR Evaluation Process: The data are adequate for the evaluation process and are presumed relevant for humans. They show a direct effect on the ovary, although not uniquely so. They add little to mechanistic understanding. They do, however, expand the list of affected species.

Sekiguchi et al. *(123)*, supported by the Cooperative System for Supporting Priority Research in Japan Science and Technology Corporation, evaluated number of ova ovulated in response to 15 or 30 international units (IU) equine chorionic gonadotropin as an assay of female reproductive toxicity in F344 rats. DEHP was used as an illustrative chemical in their system. The equine chorionic gonadotropin was injected intramuscularly (im) at 25 days of age. The animals were

killed 72 hours later, and uteri and ovaries were removed and weighed. Ova were flushed from the oviducts with saline, denuded with hyaluronidase, and counted under a microscope. Because the maximum number of ova recovered in spontaneously ovulating rats was 11, superovulation was defined as the presence of more than 11 ova in the oviducts. To evaluate the effects of the test chemical, DEHP **[purity not specified]** 0 or 500 mg/kg bw/day in olive oil was given by sc injection daily from 24 to 27 days of age. There were 12 rats treated with 15 IU equine chorionic gonadotropin, 6 of which received DEHP, and 7 rats treated with 30 IU equine chorionic gonadotropin, 3 of which received DEHP. Ovulation was induced in 4 of the 6 rats that received 15 IU equine chorionic gonadotropin in the presence or absence of DEHP treatment. In rats given 30 IU equine chorionic gonadotropin, ovulation occurred in 1 of 3 rats given DEHP and 4 of 4 rats given the vehicle. This difference was described by the authors as not statistically significant, possibly due to the small number of animals **[statistical methods not described; *P* = 0.14, Fisher exact test by CERHR]**. None of the rats treated with DEHP demonstrated superovulation compared to 2 of 4 rats given 15 IU equine chorionic gonadotropin and 1 of 4 rats given 30 IU equine chorionic gonadotropin. The mean number of recovered ova was described by the authors as having been reduced by DEHP treatment (mean ± SEM 12.7 ± 7.75 (control) compared to 2.50 ± 0.85 (DEHP) in animals given 15 IU equine chorionic gonadotropin and 8.00 ± 4.69 (control) compared to 0.33 ± 0.33 (DEHP) in animals given 30 IU equine chorionic gonadotropin **[*P* = 0.22 and 0.23, *t* test by CERHR]**). The authors concluded that DEHP may have suppressed ovulation due to disrupted ovarian steroidogenesis and/or follicle growth.

Strengths/Weaknesses: The use of intact animals allows the assessment of an integrated physiologic response. This is overshadowed by the small sample sizes, the lack of appropriate statistics, and the fact that the studies did not move past simple description, and thus, add little that is new or of value to our understanding.

Utility (Adequacy) for CERHR Evaluation Process: These data are inadequate for the evaluation process, based on small sample size and inadequate statistics

A 65-week feeding study in marmosets performed at Mitsubishi Chemical Safety Institute, Ltd. *(65)* contained information on ovarian and uterine weight and histology. Because the focus of this study was on testicular effects, the study is discussed below in section 4.2.2.2.

4.2.2 Male
Studies on the male reproductive effects of DEHP in rodents often employ young animals because these animals are more sensitive to the testicular effects of phthalates. Although toxicity produced in prepubertal animals can be considered developmental toxicity, these studies attempt to characterize testicular effects that are not unique to the developing gonad and are included in the reproductive toxicity section. There is a single study in which prepubertal boars were treated, and this study is considered in this section as well.

4.2.2.1 Cultured cells and tissues
Dees et al. *(124)*, supported by NIEHS, reported the effects of MEHP on MA-10 cells in culture. The MA-10 cell is a mouse Leydig tumor cell that resembles the normal Leydig cell in ultrastructure and in steroid synthesis, except that it produces progesterone instead of testosterone. The cells were grown for 24 hours in the presence of MEHP **[purity not specified]** 0, 0.3, 1, 3, 10, 30, 100, 300, 1000, 3000, and 10,000 μM **[0, 0.08, 0.28, 0.84, 2.8, 8.4, 28, 84, 279, 838, and 2794 mg/L]**. After MEHP exposure, cells were washed and incubated for 2.5 hours with human chorionic gonadotropin (hCG) 50 ng/mL in the absence of MEHP. Media were assayed by RIA for progesterone. Cell protein was quantified, and cell viability was assessed by

reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT). Electron microscopy was performed and cells photographed at 6000× for morphometric analysis based on nuclear and cytoplasmic volumes as a percent of total cell volume. Statistical analyses were performed by ANOVA with post hoc Tukey test. **[The methods state that cells were grown to confluence in 24-well plates and that there were 2 experimental replicates. A figure legend indicates n = 16 from 5 different experiments for progesterone and protein levels and n = 9 from 3 different experiments for cell viability using MTT determination.]**

Progesterone production was stimulated by hCG in cells not exposed to MEHP. Significant reductions in progesterone production occurred after incubation with MEHP at 3, 10, 3000, and 10,000 μM, but not at the intervening concentrations. Viability and protein were decreased at 3000 and 10,000 μM. MEHP treatment was associated with an increase in cytoplasmic lipid droplets at MEHP concentrations of 1 μM and higher. Morphometric analysis did not show alterations in nuclear or cytoplasmic volumes up to an MEHP concentration of 1000 μM, the highest tested concentration at which viability was not affected. Abnormal mitochondria, including pale, swollen, and ring-form mitochondria and mitochondria with longitudinal or degenerating cristae, were seen at MEHP concentrations of 1 μM and higher. Rough endoplasmic reticulum was decreased at concentrations of 100 and 1000 μM. The authors concluded that these data were consistent with an MEHP effect on Leydig cell mitochondria and steroidogenesis, with consequent cholesterol accumulation resulting in an increase in lipid droplet accumulation.

Strengths/Weaknesses: This report is useful for having performed an in-depth analysis of structural alterations in an immortalized cell line that produces steroids, and for confirming previous reports. A weakness is that only an integrated biochemical measure was taken (progesterone secretion), and no other probes were used to explore the site or nature of the biochemical disruption, or to confirm possible mechanisms for the observed changes (mitochondrial changes, increased lipid droplets). The authors could offer no convincing explanation for the peculiar dose-response curve**.**

Utility (Adequacy) for CERHR Evaluation Process: This paper is largely confirmatory of other studies. The model is modestly relevant (all immortalized cell lines have some significant biochemical changes which confer immortality, and thus separate them from the rest of life) providing little new knowledge.

Kang et al. *(125)*, supported by the Korean Ministry of Environment, evaluated the effect of DEHP on gap junctional intercellular communication in cultured mouse Sertoli cells obtained from normal 11–13-day-old mice. **[Most of the text indicates TM4 cells, but the abstract, a figure legend, and the discussion indicate TM5. The Expert Panel assumes based on the endpoints being studied that the TM4 cell was used. The study authors present this paper as primarily an investigation of altered gap-junctional communication as a possible mechanism of DEHP carcinogenicity, but discuss possible importance of gap-junctional communication for Sertoli–germ cell interactions.]** A neutral-red uptake assay for cytotoxicity was performed using 24-hour exposures to DEHP concentrations of 0, 1, 10, 100, 250, 500 and 1000 μM **[0, 0.39, 3.91, 39.1, 97.7, 195.3, and 390.6 mg/L]**, showing the highest concentration tested not to be cytotoxic. DNA content of the lysed cultured cells increased after a 3-day exposure to DEHP 500 μM **[195.3 mg/L; the figure legend, however, says 100 μM (39.1 mg/L)]**, interpreted as an indicator of cell proliferation. Gap junctional communication was evaluated using the scrape-loading dye transfer technique in which Lucifer yellow was added to the culture medium and scrape-loaded into the cells using a single pass of a razor blade across the cells, thus injuring some cells and allowing dye entry. This procedure was performed on a monolayer of cultured cells that had been exposed for 24 hours to DEHP 0, 100, or 500 μM **[0,**

39.1, or 195.3 mg/L]. The distance the dye traveled in 3 minutes, observed using fluorescence microscopy, was evaluated as decreased at both concentrations of DEHP compared with the control **[data not shown; amount of decrease not specified]**. Morphologic nuclear apoptotic changes in response to 12 hours of serum deprivation were assessed using the stain Hoechst 33258. Cells pretreated with DEHP 500 μM **[195.3 mg/L]** for 24 hours showed less nuclear staining than control cells, implying a reduction in apoptosis **[data not shown]**. Western blot analysis was used to quantify phosphorylated connexin-43, a gap-junction protein, and poly(ADP-ribose) polymerase (PARP), a substrate for caspase in the apoptosis pathway. Evaluation of phosphorylated connexin-43 protein was performed in lysed cells after exposure to DEHP 500 μM **[195.3 mg/L]** for 1, 4, or 9 hours. The DEHP exposure was described as reducing phosphorylated connexin-43 in a time-dependent manner **[graph shown without error bars or statistical analysis]**. Evaluation of PARP cleavage products was performed in response to 12 hours of serum deprivation in cells exposed to DEHP 500 μM **[195.3 mg/L]** for 9, 12, or 24 hours. Whereas control cells showed an increase in cleavage products with time, these products were largely absent in DEHP-exposed cells **[graph shown without error bars or statistical analysis]**. The study authors concluded that DEHP inhibited gap junctional communication, possibly associated with a decrease in phosphorylated connexin-43. A decrease in apoptosis associated with a decrease in PARP cleavage was believed possibly to be involved in the carcinogenicity of DEHP.

Strengths/Weaknesses: The weaknesses of this paper include numerous internal inconsistencies that make it difficult to ascertain what was actually done, a lack of statistical analysis, and uncertainty about experimental rationale and execution. Dye transfer experiments frequently require more than 3 minutes for the transfer of the dye to adjacent cells. The nuclear staining and early indicators of apoptosis are generally considered as issues separate from those relating to cell communication, so the combination here is perplexing. Sertoli cells are not targets of the tumorigenic activity of DEHP, so are a model of questionable relevance to the in vivo situation. The working dose most frequently used in this study was exceptionally high, and while perhaps useful as a biochemical tool, is of limited relevance.

Utility (Adequacy) for CERHR Evaluation Process: This study is not useful for the evaluation process, as there is considerable uncertainty about what was done, whether statistical analysis was performed, and the relevance of the findings to the in vivo situation.

Suominen et al. *(126)*, supported by the EU and the Academy of Finland, evaluated the effects of MEHP on segments of CD-1 mouse seminiferous tubules. Testes from 2–3-month-old mice were decapsulated and microdissected to provide 1 and 2 mm tubule segments at stages III–V, VII–VIII, and IX–XI (determined by transillumination and confirmed in squash preparations). Tubule segments at stages IX–XI were incubated for 24 hours with MEHP **[purity not specified]** at concentrations of 0, 0.01, 0.1, and 1.0 mM **[0, 2.8, 28, and 279 mg/L]** for 24 hours. Tritiated thymidine was added for the last 4 hours of incubation for estimation of DNA synthesis by thymidine incorporation. Graphic representation of the incorporated label at the end of the experiment suggested a concentration-related decrease in DNA synthesis, but none of the values was statistically different from the control by ANOVA. Incubation of 2 mm segments of tubules at stages III–V and VII–VIII with MEHP 0.1 mM **[28 mg/L]** also had no significant effect on thymidine incorporation. Stage IX–XI tubule segments were incubated for 8 hours with MEHP at 0, 0.01, 0.1, and 1.0 mM **[0, 2.8, 28, and 279 mg/L]**, following which squash preparations were evaluated for apoptosis using in situ 3′ end-labeling followed by light microscopy to count positive cells. A subsequent time-course experiment was performed in which stage IX–XI tubules segments were incubated with MEHP 0.1 mM **[28 mg/L]** for 4, 8, or 24 hours and evaluated for apoptosis by *in situ* 3′ end-labeling. Stage specificity of apoptosis induction was evaluated using

segments of tubules at stages III–V and VII–VIII incubated with MEHP 0.1 mM **[28 mg/L]** for 8 hours. Statistical evaluation used ANOVA with post hoc Dunnett test for dose-response experiments and *t* test for comparison of stage-specific results with one another. The number of apoptotic cells per tubule segment was approximately doubled **[estimated from a graph]** by MEHP 0.01 and 0.1 mM **[2.8 and 28 mg/L]** compared to control but was not significantly altered by MEHP 1.0 mM **[279 mg/L; lack of effect attributed by the study authors to a high incidence of early cell death at this concentration]**. In the time-course experiment, MEHP 0.1 mM **[28 mg/L]** significantly increased the number of apoptotic cells per tubule segment only at 8 hours. **[The numeric value appeared to be higher from a graph at 24 than at 4 or 8 hours, but the standard error was large, and statistical significance was not identified.]** In the stage-specificity experiment, only tubule segments at stages IX–XI showed an increase in number of apoptotic cells after MEHP exposure. The authors concluded that their finding of stage specificity in the mouse was "partly in agreement" with previous reports that stages XI–XIV and I–II were the most sensitive to phthalate testicular toxicity in the rat, associated with the reliance of these stages on FSH and with inhibition by phthalates of FSH binding.

Strengths/Weaknesses: One strength of these studies is that they were performed with technical competence and were not over-interpreted. No biochemical mediators of cell death or stage-specific biochemistry were evaluated in these studies, which is something uniquely available in this type of preparation.

Utility (Adequacy) for CERHR Evaluation Process: These studies appear adequate for the Evaluation process, but add little that is new to our understanding of where and how DEHP/MEHP works in the testis.

Andriana et al. *(127)*, supported by the Japanese Ministry of Health, Labour, and Welfare, evaluated morphologic MEHP-associated alterations in spermatogenic cells in Sprague-Dawley rat testis culture. Testes were harvested from an unspecified number of 20-day-old animals and 1 mm^3 portions were cultured on filter paper. MEHP **[purity not specified]** was added to culture media at 0, 10^{-6}, 10^{-3}, 1, or 100 µM **[0, 0.00028, 0.28, 279, or 27,936 mg/L]**. Testis fragments exposed at each concentration were evaluated at 1, 3, 6, and 9 hours. TUNEL staining using a commercial kit was applied to 5 µm paraformaldehyde-fixed paraffin-embedded sections with methyl green counterstaining. Apoptotic cells were counted in 25 randomly selected round tubules from 20 tissue cultures, expressed as a percentage, and compared using ANOVA. Transmission electron microscopy was used to characterize ultrastructural evidence of apoptosis and necrosis. Tissues for electron microscopy were fixed in 2.5% glutaraldehyde/0.05 M cacodylate buffer, post-fixed in osmium tetroxide, and embedded in Araldite-M. **[No quantitative results were presented from the transmission electron microscopy portion of the study.]** In the control cultures, apoptosis increased after 9 hours of culture with 2.8% of cells positive. In the MEHP-treated cultures, apoptosis increased in a time- and concentration-dependent manner, with a maximum of 14% of cells affected in the 100 µM culture at 9 hours (Figure 3). At the highest concentration, there was evidence of Sertoli cell apoptosis at 1 hour, characterized by the authors as partial lysis of the nuclear membrane by transmission electron microscopy. Partial lysis of spermatogenic cell nuclear membranes was noted at 1 hour at the 1 and 100 µM MEHP concentrations. The authors concluded that "even a low concentration of MEHP caused permanent changes in testicular tissue cultures of rats."
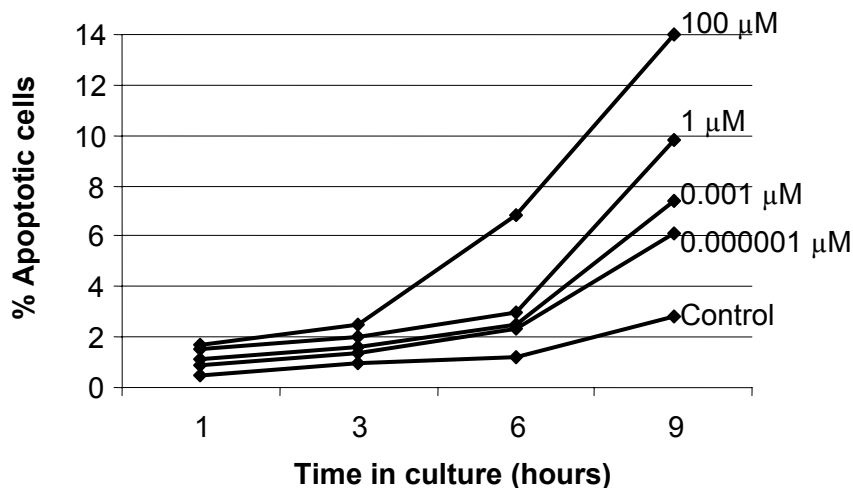
Figure 3. Percent Apoptotic Cells in Rat Testicular Culture with MEHP

Drawn from data in Andriana et al. *(127)*. Standard error bars omitted for clarity. Statistical comparisons were not indicated in the original.

Strengths/Weaknesses: One strength of this design was the novel exposure system, which allowed for normal cell-cell interactions to be maintained. Another unique aspect was the wide range of concentrations used. One weakness is the unspecified purity of the MEHP, and also the fact that all tubules were not affected equally by MEHP, so the selection of random tubules for assessment depended on truly random selection, which would almost certainly have diluted the effect of the MEHP. Another weakness is the uncertainty surrounding the use of electron microscopy-derived membrane structure to reach conclusions about cell death, and the fact that this finding is inconsistent with the published in vivo literature (i.e., Sertoli cell death is not reported after in vivo dosing and evaluation). The use of the word "permanent" when describing effects of a 9-hour exposure is questionable.

Utility (Adequacy) for CERHR Evaluation Process: This study appears adequate for the evaluative process, although it produces no new insights into the site or mechanism of MEHP toxicity, confirming what was known previously.

Andriana et al. *(128)*, supported by the Japanese Ministry of Health, Labour, and Welfare, evaluated the effects of MEHP **[purity not specified]** on cultured goat testis. Testes were harvested from 2-month-old Shiba goats, decapsulated, and cut into 1-mm^3 pieces for culture on filter paper floated in Dulbecco's Minimal Essential Medium. The medium contained antibiotics, DMSO, and ethanol. Some cultures included MEHP at 0.001, 1, or 100 nmol/mL **[0.0028, 2.8, or 279 mg/L (nmol/mL = µM); DMSO and ethanol concentrations, control culture conditions, number of animals, and number of cultures were not specified]**. Explants were harvested at 1, 3, 6, or 9 hours of culture and fixed in 2.5% glutaraldehyde/0.05 M cacodylate buffer. Specimens were prepared for light microscopy using toluidine blue staining or transmission electron microscopy using uranyl acetate and lead citrate. Results were expressed qualitatively. Light

microscopy showed a concentration-related increase in apoptotic or necrotic Sertoli and spermatogenic cells with vacuolization and sloughing of germ cells beginning at 3 hours "at each concentration." Transmission electron microscopy showed infrequent distended mitochondria, abnormal nuclear vesicles, and ruptured mitochondrial membranes, which were apparent as early as after1 hour of exposure to 1 μM MEHP **[the results are not specific about which cells were affected, but the figure legend and discussion suggest that the Sertoli cells were affected first]**. At 3 hours, vacuolization and abnormal vesicles were described as frequent, with damage in Sertoli and germ cells. At 6 hours, there were apoptotic spermatogonia. The lowest concentration (0.001 μM) produced marginated chromatin in Sertoli cells at 6 hours. The authors concluded that even the lowest MEHP concentration produced permanent testicular damage, and that their results support the Sertoli cell as the primary target of MEHP toxicity, with germ cell damage as a consequence of Sertoli cell alterations.

Strengths/Weaknesses: A strength of this paper is that it adds another species to the list of those shown to be affected by DEHP/MEHP, and it uses methods shown to have worked for the more commonly employed rat model. It avoids having to expose the whole goat, which saves compound and avoids unknown toxicokinetic issues. Significant weaknesses are the uncertainty about control culture conditions, number of animals used, and the number of experiments, not to mention the uncertainty introduced by including the solvents DMSO and ethanol in the medium. While paucity of experimental detail limits confidence in the relevance of these data, confidence is further decreased by the solvent issues because DMSO can significantly enhance the toxicity of lipophilic compounds in vitro. There is uncertainty about the relevance of the findings at the lower dose levels because these concentrations in vivo have not been previously associated with the reported effect.

Utility (Adequacy) for CERHR Evaluation Process: This study is of limited relevance and utility to the evaluation process, based on the uncertainty about the methods, solvents, and the doubt around the results at the lowest concentrations, and the assertions of "permanent" damage after short-term in vitro exposure and asssessment.

Awal et al. *(129)*, supported by the Japan Society for Promotion of Sciences and the Japanese Ministry of Health, Labour, and Welfare, evaluated the effects of MEHP on guinea pig seminiferous tubule culture. Tubules harvested from 28-day-old animals were exposed to MEHP **[purity not specified]** in corn oil at 0, 1, 10, and 100 μM **[0, 279, 2794, or 27,936 mg/L]** for 3, 6, or 9 hours. Cultured tissues were evaluated by light and transmission electron microscopy and by in situ TUNEL staining for apoptosis. Intact round seminiferous tubules (100 per dose group) were counted for apoptotic spermatogenic cells, expressed as a percent. Statistical analysis was by ANOVA with the Fisher least significant differences test. After 3 or more hours of MEHP exposure, germ cell detachment was evident by light microscopy, and Sertoli cell vacuolation was identified by electron microscopy **[effect levels not given, but the figures show tubule sections from the 100 μM group, for which the response was said to be maximal]**. The number of apoptotic cells by TUNEL staining was said to increase with exposure level and time. **[The data figure does not have significant differences marked; the legend says that significance between treated and control cultures is recognized at *P* < 0.05.]** The authors concluded that "MEHP induces testicular toxicity in guinea pigs in vitro."

Strengths/Weaknesses: It is unclear whether the animals were treated with MEHP before donating tissues to culture or whether the tissues were treated in vitro and exposed to MEHP dissolved in corn oil and added to the culture. There is sufficient confusion about the wording to make the method quite unclear, although much of the description seems to indicate the latter (corn oil was added to the cultures). Weaknesses of this study include this confusion, the unusual

exposure paradigm in the absence of any determination of MEHP levels in the medium, uncertainty in the paper about which effects were seen at which concentrations, and the implausibility of finding these effects in cultured tissue fragments due to highly lipophilic compounds being dosed into an aqueous medium in corn oil. The only light-level micrographs use different magnifications for the control and treated cultures, making it difficult for the reader to closely assess the adequacy (i.e., normal structure) of the control cultures. The study confirms an affected cell type but adds little that is detectably new to our understanding.

Utility (Adequacy) for CERHR Evaluation Process: This study is inadequate for the evaluation process due to the confusion about the exposure and the fact that the data are redundant with previous literature.

4.2.2.2 In vivo studies with or without ex vivo component

Richburg et al. (130), supported by NIEHS, NIH, and the Burroughs Wellcome Fund, evaluated the effect of MEHP on testicular apoptosis in *gld* mice, which have a nonfunctional form of fas-ligand (fasL). The study was performed to explore the involvement of the Fas Ligand, a mediator of cell-cell death, and hypothesized to mediate germ cell death after Sertoli cell damage, in the development of the DEHP lesion in mice. **[The study also evaluated radiation-induced injury; only the MEHP results are presented here.]** Wild-type controls were C57BL/6 males at 28 days of age. Wild-type and *gld* mice were given MEHP (>94% purity) in corn oil at 0 or 1000 mg/kg as a single gavage treatment. The animals were killed 0, 3, 6, 12, of 24 hours later and testes were harvested. One testis was fixed in 10% neutral buffered formalin embedded in glycol methacrylate, sectioned, and stained with periodic acid-Schiff/hematoxylin for light microscopy. The other testis was frozen until analyzed for apoptosis using a commercial TUNEL staining kit. Apoptosis was evaluated (3 animals/group) using the number of tubules with 0–3 or >3 apoptotic germ cells. Homogenization-resistent testicular sperm heads were also counted. Statistical comparisons were made using ANOVA with Fisher least significant difference test. Baseline testicular parameters (n=15 *gld* and 17 wild-type mice at time 0) showed the *gld* mice to be 15% heavier and to have 6% higher testis weights than wild-type mice. There were 17% more sperm heads/testis in the mutants, which also had 2.2 times the proportion of tubules with >3 apoptotic germ cells. After MEHP treatment, there was histologic evidence of injury (germ cell sloughing, increased tubule lumen, Sertoli vacuolation) in both *gld* and wild-type mice, but only the wild-type mice had histologic findings consistent with germ cell apoptosis (cell shrinkage, chromatin condensation). TUNEL labeling increased in wild-type mice 3–12 hours after MEHP treatment, with the proportion of tubules containing >3 apoptotic germ cells increasing 2.7-fold in that interval **[estimated from a graph]**. By contrast, there was only a 1.7-fold increase in tubules containing >3 apoptotic germ cells in the *gld* mice at 12 hours **[estimated from a graph]**. The proportion of tubules meeting these criteria was statistically higher in wild-type than mutant mice at 6, 12, and 24 hours. The authors concluded that the insensitivity of *gld* mice to MEHP-induced germ cell apoptosis "further underscores the participation of the [f]as system in the regulation of germ cell apoptosis after MEHP-induced Sertoli cell injury."

Strengths/Weaknesses: Strengths of this study are the technical competence with which it was performed and the creativity of the approach to address the experimental question. The data are internally consistent and support the proposed hypothesis. The study used MEHP as a tool to show the involvement of fasL in a lesion.

Utility (Adequacy) for CERHR Evaluation Process: These data are adequate for the evaluation process, in that they appear to define the mechanism by which the germ cells die. These data were not intended to shed any light on the means by which MEHP affects Sertoli cells.

Ichimura et al. (131), support not indicated, evaluated the expression of fas, fasL, and caspase-3, which are 3 apoptosis-associated proteins, as well as TUNEL positivity and electrophoretic DNA laddering in the testes of DDY mice treated with DEHP. DEHP **[purity not specified]** in corn oil was given orally **[gavage assumed]** to 4-week-old male mice as a single dose of 0, 4, 40, 400, or 4000 mg/kg bw (n=3/dose group) **[doses given in the original in mg/g and converted by CERHR to mg/kg by multiplying × 1000]**. Testes were removed 12 hours after the treatment. One testis per animal was fixed in paraformaldehyde and sectioned for immunohistochemistry for fas, fasL, and caspase-3. TUNEL labeling was evaluated in sections as an indicator of apoptosis. DNA extraction and electrophoresis was performed on homogenates of both testes from 3 other animals, with the density of electrophoretic bands estimated photographically using Adobe PhotoShop® software. FasL positivity was identified in a distribution consistent with Sertoli cell cytoplasmic processes and associated spermatocytes. Fas and caspase-3 co-localized in the middle to outer portion of the epithelium. **[Results are described for the group given DEHP 4000 mg/kg bw.]** TUNEL-positive nuclei were clustered in the middle layer of the epithelium. The maximum number of positive nuclei per section in the 0, 4, 40, 400, and 4000 mg/kg bw groups were 3, 3, 5, 7, and 22, respectively. **[Statistical comparisons were not presented, and numbers of total cells/section were not given.]** DNA laddering was identified in all dose groups, with band density estimated as 2.2 times the control in the high-dose group and 1.1 times the control in the other dose groups. **[Statistical comparisons were not presented.]** The authors interpreted the numeric increase in number of TUNEL-positive nuclei per section as possibly indicating a dose-response relationship, concluding that exposure to DEHP at as little as 40 mg/kg bw might produce an increase in apoptosis in mouse testis. Further, they concluded that the 10% increase in electrophoretic band density in the lower dose groups meant that DEHP might have an effect on mouse testis after a dose as low as 4 mg/kg bw.

Strengths/Weaknesses: The strengths of this study include the technical co-localization approach and the number of doses of DEHP used. Weaknesses include the means of quantifying the density of bands after electrophoresis.

Utility (Adequacy) for CERHR Evaluation Process: This study is adequate for the evaluation process, although it does not add significantly to our understanding of the early events in the production of the testis lesion, nor was it intended to. The point of this study was to examine the co-localization of factors involved in cell death after treatment with a compound that would predictably induce that death. The changes at the lowest two doses, although detectable, are probably of little biological consequence.

Giammona et al. (132), supported by NIEHS, examined the role of MEHP in inducing apoptosis in pubertal rodent testicular germ cells and evaluated the role of fas-independent receptors in apoptosis. A single dose of 1000 mg/kg bw MEHP (> 97% purity) in corn oil was administered by gavage to 5-week-old Sprague-Dawley rats, 28-day-old wild-type C57CL/6 mice, or 28-day-old *gld* mice. The *gld* mice express a dysfunctional fasL protein, which cannot bind to the fas receptor to initiate apoptosis. A commercial TUNEL staining kit was used to identify apoptosis. Immunohistochemistry was used in sections of rat testis to identify fas and DR4, DR5, and DR6 receptors, which are fas-independent death receptors in the tumor necrosis factor (TNF) superfamily. Western blot was used to measure caspases, fas receptor, and DR receptors in homogenized mouse and rat testis. **[Statistical analysis methods were not described.]** Following MEHP exposure, apoptosis was found to occur primarily in spermatocytes in both wild-type and *gld* mice. In wild-type mice, germ cell apoptosis was significantly increased from 6 to 48 hours following MEHP exposure. Apoptotic activity peaked between 12 and 24 hours with ~5-fold increases compared to baseline levels. A significant ~2-fold increase in apoptosis compared to baseline levels was observed at 12 and 48 hours following MEHP exposure of *gld*

mice. In both groups of mice, apoptotic activity returned to baseline levels by 96 hours following exposure. Western blot analyses revealed that fas expression significantly increased in wild-type mice (~3-fold) at 3 hours following MEHP exposure. There was no significant alteration in fas expression following MEHP exposure in *gld* mice. Expression of DR4, DR5, and DR6 proteins occurred in both wild-type and *gld* mice, but MEHP exposure did not increase expression in either strain. DR5 but not DR4 expression significantly increased in Sprague-Dawley rat testes (~1.5-fold) at 1.5 and 3 hours following MEHP exposure. Procaspase 8 cleavage products, downstream receptor-mediated signals of apoptotic pathways, were detected in testes of wild-type and *gld* mice, but expression was significantly increased only in *gld* mice at 6 hours following MEHP exposure. Electrophoretic mobility shift assays demonstrated that DNA binding of NFκB, a receptor-mediated downstream signal possibly involved in cell death or survival, was generally reduced in wild-type mice but upregulated in *gld* mice following MEHP exposure. The study authors concluded that these findings demonstrate that germ-cell related death receptors and downstream signaling products appear to respond to MEHP-induced cell injury; it has not yet been determined if these factors are involved in testicular apoptosis following MEHP exposure.

Strengths/Weaknesses: The creative experimental approach and competence are both strengths of this paper. These studies were using MEHP as a tool to explore the cell death mechanism in the rat and mouse testis and were effective in charting the expression of death-related genes in germ cells after exposure to a Sertoli cell toxicant.

Utility (Adequacy) for CERHR Evaluation Process: The data are considered adequate and useful for the evaluation process, mostly by way of identifying those factors involved in germ cell death.

Dalgaard et al. *(133)*, supported by the Danish Medical Research Council, evaluated the acute effects of MEHP on the testes of 28-day-old Wistar rats in an attempt to infer initial effects on the androgen receptor or intracellular levels of cyclic AMP. A single dose of MEHP **[purity not specified]** was given in corn oil by gavage at 0 or 400 mg/kg bw. Rats given corn oil were killed 2 hours later, and rats given MEHP were killed 3, 6, or 12 hours later (n = 12 per time point). Testis sections were stained with hematoxylin and eosin for light microscopy or were immunostained for vimentin and androgen receptor. Testis sections were also TUNEL stained for determination of apoptosis. Portions of testis and ventral prostate were processed with RT-PCR for quantification of *TRPM-2* gene message, and caspase-3 was estimated in testis samples based on its interaction with a colorimetric substrate. A commercial ligation-mediated PCR kit was used to detect nucleosomal ladders in testicular tissue, and TUNEL staining was performed using a commercial kit. Randomly selected tubules were evaluated for the presence of 0, 1–3, or >3 apoptotic germ cells/tubule. Statistical comparisons were made using ANOVA with post hoc Dunnett test. Light microscopy showed germ cell sloughing beginning 6 hours after MEHP treatment. Collapse of vimentin filaments in the Sertoli cell cytoplasm and perinuclear condensation were evident by immunostaining at 3 hours and persisted throughout the study. Androgen receptor staining was not altered by treatment at any time point. *TRPM-2* expression was increased at 3 hours after MEHP treatment but returned to control levels at 6 and 12 hours. Caspase-3 activity was increased by treatment, although the authors noted that there was considerable caspase-3 activity in the control and that the 6-hour value was not statistically different from the control (Figure 4).
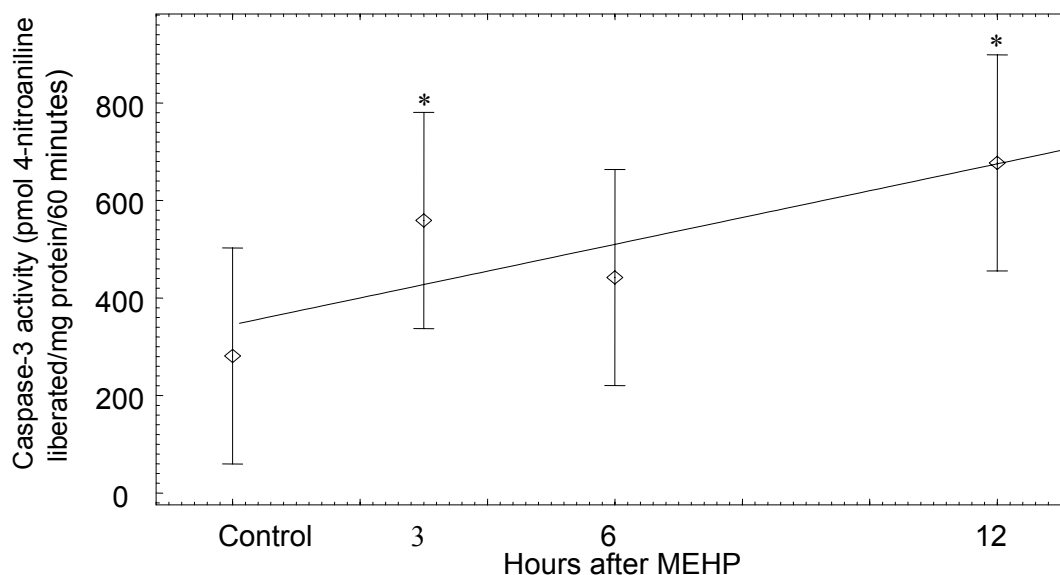
Figure 4. Testicular Caspase-3 Activity after Administration of a Single Oral Dose of MEHP to Wistar Rats

Drawn from Dalgaard et al. *(133)*. Mean ± SD, n = 8. *Different from control, P < 0.05 by ANOVA with post hoc Dunnett test.

There were no detectable treatment effects on number of apoptotic cells per tubule or DNA laddering patterns. The authors concluded that the collapse and perinuclear condensation of vimentin fibers beginning at 3 hours was an early and sensitive MEHP-associated change, occurring at an exposure level 5 times lower than previously reported. The increase in caspase-3 was consistent with MEHP initiation of apoptosis but could not be confirmed by later apoptotic events, perhaps due to the large variability in TUNEL staining and DNA laddering among individuals.

Strengths/Weaknesses: This study attempted to investigate potential early events in the lesion produced by MEHP and repeated some previous findings. A weakness is the inability to explain the discordance between some of the early events they report, and the absence of expected later events.

Utility (Adequacy) for CERHR Evaluation Process: The data are adequate for the evaluation process and eliminate some possible early events (changes in androgen receptor events or cAMP levels) as possible mediators of MEHP toxicity.

Park et al. *(134)*, supported by NIH, evaluated the role of zinc depletion as a causative factor in the testicular toxicity of DEHP in young Sprague-Dawley rats. Animals were treated beginning at 25 days of age. Dosing was by gavage with DEHP in corn oil at 0 (n=15) or 2000 (n=35) mg/kg bw daily for up to 14 days. Five control animals per time point were decapitated after 2, 7, or 14 days of treatment, and 5 DEHP-treated animals per time point were decapitated after 1, 2, 3, 5, 7, 10, and 14 days of treatment. Testes were removed and weighed, following which 1 testis was frozen for subsequent estimation of zinc-transporter-1 mRNA by branched DNA signal amplification using a commercial kit. The other testis was fixed in neutral buffered formalin. One-half of the fixed testis was sectioned and stained with hematoxylin and eosin for histologic evaluation by light microscopy and for quantification of apoptosis using the TUNEL assay. An apoptotic index was calculated based on 1000–1500 cells per animal, evaluated at 400× for the

percentage showing apoptosis. The other half of the testis was digested with nitric acid for zinc determination by atomic absorption spectrophotometry. Comparisons of testicular zinc-transporter-1 mRNA, zinc, and apoptotic index were made using ANOVA, pooling data from the 3 control time points, which did not differ from one another. Body weight comparisons, made using repeated measures ANOVA, showed a decrease in body weight gain after DEHP exposure beginning on the third day of treatment. Absolute testis weight was decreased in the DEHP group on treatment days 7 and 14 to 45 and 25% of control **[estimated from a graph]**. Relative testis weight was also significantly decreased by DEHP at these time points. DEHP treatment was associated with histologic evidence of apoptosis, which occurred early and was most marked by treatment day 3, and necrosis, which was most severe from treatment day 7. Testicular zinc did not change over the 14-day treatment period in control animals but decreased in DEHP-treated animals beginning on treatment day 3 and declining to 73% of the control level by treatment day 14 **[estimated from a graph]**. The testis level of zinc-transporter-1 mRNA was not altered by DEHP until treatment day 14, when it was about 30% lower than the control level. The authors noted that the reduction in testicular zinc associated with DEHP treatment was consistent with other studies but concluded that this reduction was likely to be secondary to DEHP testicular toxicity rather than a cause of it. They noted that zinc depletion was not demonstrated prior to treatment day 3 whereas histologic abnormalities of the testis were evident within 1 day of initiating treatment. The maintenance of normal zinc-transporter-1 mRNA until treatment day 14, well after the earliest demonstration of testicular toxicity, suggested that this transporter protein does not play a role in mediating DEHP testicular toxicity.

Strengths/Weaknesses: The technical approach and competence are two strengths of this paper. This study explored the loss of zinc as a causative factor that might be unique to the DEHP lesion. The authors clearly showed a separation between germ cell death and loss (early) and changes in the zinc levels and transporter expression, which happened after the initial germ cell changes. The authors marshal considerable previous data in support of their contention that zinc changes are probably secondary and do not mediate the germ cell death after DEHP exposure.

Utility (Adequacy) for CERHR Evaluation Process: This study is adequate for the evaluation process and is useful in providing a summary of, and some recent data on, zinc changes in the testis and their relationship to the DEHP-induced lesion. Collectively, the data suggest that zinc loss is not a causative factor in this lesion.

Kasahara et al. *(135)*, supported by the Japanese Ministry of Education, Culture, Sports, Science, and Technology, evaluated the role of oxidative stress in DEHP-associated testicular toxicity in Wistar rats. In the first experiment, males at 4–5 weeks of age were given DEHP **[purity not specified]** in corn oil by gavage at 0, 1000, or 2000 mg/kg bw/day (n=8–15/group) for 7 days. Animals were killed, and body weight and weights of testis, liver, and kidneys were determined. Total glutathione, low-molecular-weight thiols, and ascorbic acid content of testis, liver, and kidney were determined, and in 5 animals/group, glutathione peroxidase and catalase were estimated in these tissues. Body and kidney weights were decreased by about 20% **[estimated from a graph]** by 2000 mg/kg bw/day DEHP. DEHP 1000 mg/kg bw/day increased liver weight by about 50% and decreased testis weight by about 20%, with an additional 50% decrease in testis weight at 2000 mg/kg bw/day **[estimated from a graph]**. Total glutathione, low-molecular-weight thiols, and ascorbic acid were decreased in testis after treatment with DEHP 1000 mg/kg bw/day. Glutathione and low-molecular-weight thiols were increased, and ascorbic acid was unchanged in liver and kidney at both DEHP dose levels. These antioxidants were unchanged in plasma and brain of treated animals **[data not shown]**. Glutathione and catalase were increased in testis at both doses of DEHP and unchanged or decreased in liver and kidney.

Testicular cells were obtained from minced testis, and differential centrifugation was used to obtain predominantly spermatocytes. These cells were incubated with a chemiluminescent probe and digitonin, following which 5 mM NADH was added to initiate release of reactive oxygen species. The release of reactive oxygen species was measured as increased chemiluminescence that was reported to increase in a time-dependent manner in cells derived from animals treated with DEHP 2000 mg/kg bw/day. **[The data graph is not marked with significant differences.]** The increase in chemiluminescence was prevented by addition of superoxide dismutase, catalase, or sodium azide, suggesting to the authors that the superoxide radical and hydrogen peroxide were the reactive oxygen species responsible for the increased chemiluminescence. Reactive oxygen species generation was also evaluated in Sertoli cell and germ cell cultures obtained from 14-day-old rats. Cultures were grown in the presence of DEHP or MEHP **[the MEHP was synthesized and HPLC-confirmed to be minimally contaminated]** at concentrations of 0, 25, 50, 100, and 200 µM, using fluorescence of 2′,7′-dichlorofluorescein to estimate reactive oxygen species. Fluorescence in germ cells was increased by MEHP in a dose-dependent manner beginning at 50 µM **[19.6 mg/L]**. There was no increase in fluorescence at any concentration of DEHP in germ cells and no increase in fluorescence in Sertoli cells at any concentration of either chemical.

Testicular sections were stained using the TUNEL method 12 hours after administration of DEHP 2000 mg/kg bw **[it is not stated whether these were animals from the first experiment or if this experiment involved new animals given a single dose of DEHP]**. TUNEL-positive cells were reported to be markedly increased in the testes of DEHP-treated animals, and TUNEL-positive cells showed chromatin condensation after propidium iodide staining, confirming apoptosis **[no data were shown for TUNEL staining or propodium iodide staining]**.

In a final experiment, mitochondria were isolated from fresh testes **[age of animals not specified, n = 3 indicated in data figure]** and incubated with DEHP or MEHP at concentrations of 0, 2, 10, or 50 µM for 5 minutes. Samples were centrifuged at 8000 *g* for 5 minutes, and supernatants were evaluated using SDS-PAGE and Western blotting for cytochrome *c*. The authors described an increase in cytochrome *c* release from mitochondria exposed to MEHP but not DEHP **[the data figure is not marked with significant differences]**.

The authors concluded that DEHP treatment of rats causes testicular atrophy through generation of reactive oxygen species and reduction in antioxidant enzymes. MEHP-associated apoptosis in germ cells was attributed, at least in part, to release of cytochrome *c* from mitochondria in response to oxidative stress.

Strengths/Weaknesses: The conclusions from these extensive studies are minimally but measurably reduced by the uncertainties noted above. The variety of approaches used for these studies is a clear strength. The report is weakened by the use of whole-testis mitochondria, which is a problem, given that the testis is composed of DEHP-responsive cells and DEHP-resistant cells, at least as far as oxidative response is concerned. Of greater significance are the data indicating that germ cells are affected but not Sertoli cells; this finding contradicts the existing gestalt in the field, supported by numerous histologic studies that show changes in Sertoli structure prior to germ cell death. The localization data, combined with the time course of apoptosis in vivo and active oxygen generation (Figs 7 and 4B, respectively, of the report), make it difficult or impossible to conclude that the oxidative damage is a cause of the germ cell death. Additionally, several of the time-dose points have a great deal of cell death occurring, making this separation impossible. Thus, it is likely that oxidation occurs as part of the cell death process in dying germ cells.

Utility (Adequacy) for CERHR Evaluation Process: These data are adequate for the evaluation process, but the uncertainties over which comes first, the oxidative damage or the cell death, limit

their mechanistic utility. The data showing the presence of oxidative damage are clear and believable; the uncertainties lie with the assertion that this damage produces rather than results from the germ cell death.

Ablake et al. *(136)*, supported by the Japanese Ministry of Education, Science, Sports, and Culture, evaluated the effect of vitamins C and E in accelerating recovery from the testicular toxicity of DEHP in CD mice. Ten-week-old males were given 0 or 2% dietary DEHP (>98% purity) for 16 days following which all animals were given untreated feed for an additional 50 days. A vitamin-supplemented group was given drinking water containing vitamin C 3.0 mg/mL and vitamin E powder 1.5 mg/mL during the 50-day recovery period. **[Based on the reported estimated mean feed consumption of 5 g/mouse/day and mean fluid consumption of 5 mL/mouse/day, and using the starting mean body weight of 35 g/mouse (estimated from a graph), CERHR calculated a mean DEHP intake during the treatment period of 2857 mg/kg bw/day. During the recovery period, the mean intake of vitamin C was calculated at 429 mg/kg bw/day and the mean intake of vitamin E at 214 mg/kg bw/day.]** Counting the first day of DEHP treatment as day 0, 4–7 animals/treatment group were killed on each of days 0, 3, 5, 7, 9, 12, 15, 25, 35, 45, 55, and 65. **[The Expert Panel notes that time points after day 15 did not include DEHP treatment and included supplemented vitamins C and E.]** Testes were harvested and samples prepared for light microscopy, electron microscopy, TUNEL staining, BrdU incorporation, or histochemical detection of lipid peroxidation. Light microscopy was performed on sections fixed in Bouin solution, embedded in plastic, and stained with hematoxylin and eosin. Spermatogenic disturbance was graded in at least 100 tubule sections per mouse using a 10-point scale, and the ranks were averaged to produce an overall score per mouse. Electron microscopy, TUNEL staining (using a commercial kit), BrdU incorporation, and detection of lipid peroxidation were performed on samples obtained on days 0, 7, and 15 (before, in the middle, and at the end of the DEHP treatment period). Statistical analysis was by ANOVA.

Body weight decreased during the 16 days of DEHP exposure, recovering to control levels 10 days after DEHP was removed from the feed. Absolute testis weight increased on day 5 of DEHP treatment, and testes appeared grossly edematous. By day 12, testis weights were significantly decreased by DEHP treatment. Testis weight recovered toward control levels 20 days after the cessation of DEHP treatment, although at the end of the experiment on day 65, testis weight had not completely recovered. Testis weight was higher in the group given vitamin supplementation than in the group not given vitamin supplementation during the recovery period **[although pair-wise statistical comparisons of these 2 groups were not reported]**. Relative testis weight showed a pattern similar to that of absolute testis weight. The mean histologic scores declined during the period of DEHP treatment from 10 to 2 **[estimated from a graph]**. The scores returned toward control levels after cessation of DEHP treatments with the vitamin supplemented group reaching nearly control levels by day 45 and the unsupplemented group reaching nearly control levels by day 55. There were significant differences between supplemented and unsupplemented DEHP-treated animals in histologic score on experiment days 25, 35, and 45. Electron microscopy during the DEHP treatment period showed vacuoles and dark droplets in Sertoli cell cytoplasm in contrast to light microscopic findings, which apparently did not show Sertoli cell damage. Lipid peroxidation was prominent in the basal compartment of the seminiferous epithelium and surrounding interstitial tissues on day 15 of treatment. A few cells with TUNEL staining and BrdU staining were noted on day 15, but these evaluations appeared limited due to the hypocellularity of tubules in day 15 DEHP-treated animals. The authors concluded that the antioxidant effects of vitamins C and E accelerated the recovery of the testis from DEHP-induced injury.

Strengths/Weaknesses: The novel design of this study is a strength. This study really tests the ability of the testis to recover after DEHP-induced damage, and it is not clear that the DEHP

damage is significantly different from any other toxicant-induced lesion. The findings are weakened by the lack of measurement of feed and water intake, and the consequent inability to report actual DEHP or vitamin consumption, the lack of data on the levels of these two antioxidants in the mouse chow, and the lack of verification of water levels of these two vitamins (vitamin E is not inherently water-soluble, making this verification more important). The authors concluded that oxidative damage is the mechanism of the DEHP lesion, which is consistent with previous literature they cite, but cannot be a logical conclusion of this paper because they did not test for abrogation of the DEHP lesion by concurrent vitamin and DEHP administration. The DEHP-specific conclusion is also significantly limited by the fact that only a single toxicant was used. Had the authors employed another testicular toxicant that works by a presumably different mechanism, they might have seen a difference, in which case a DEHP-specific conclusion would be warranted. It would also have been very useful for the authors to have evaluated this finding in another species to address its extensibility.

Utility (Adequacy) for CERHR Evaluation Process: These data are adequate for the review process, as the study was conducted in a credible way. The data are of use in considering treatments for recovery, but are of limited use in determining mechanisms of DEHP toxicity.

Kijima et al. *(137*), support not indicated, administered a single oral dose of DEHP (99% purity) in corn oil **[gavage assumed]** to 6-week-old male Crj:CD(SD)IGS rats at dose levels of 0, 20, or 2000 mg/kg bw. This study was conducted to evaluate expression changes in many genes, looking for new leads on mechanism. Rats were killed 3, 6, 24, or 72 hours after dosing and testes were excised. There were 6 rats per dose group and time point. The right testis of each rat was fixed in 4% paraformaldehyde, embedded in paraffin, sectioned at 5 μm, and stained with hematoxylin and eosin or used for TUNEL staining. The left testis was frozen at −80ºC for subsequent RNA extraction. RNA extracts were pooled within dose and time groups and analyzed using a microarray consisting of 3060 cDNA probes. Altered regulation was defined as expression at less than half or more than twice the control level for the microarrays. Real-time PCR was used to evaluate mRNA expression for the apoptosis-related genes *caspase-2*, *-3*, *-6*, *-8*, *-9*, and *-11*, *bcl-2*, *bax*, *fas*, and fasL, normalized to β-actin. Statistical analysis was performed using the Student *t* test. There were no treatment-related histologic changes, but apoptotic cells were identified by TUNEL staining near the seminiferous tubule basement membrane in testes from rats treated with DEHP 2000 mg/kg bw. At 3 and 6 hours, the apoptosis involved spermatogonia, and at 24 hours the apoptosis also involved spermatocytes and spermatids. At 72 hours after exposure, only spermatogonia and spermatocytes were affected. The proportion of tubules with at least 3 apoptotic cells was increased significantly compared to control at 24 and 72 hours after treatment with DEHP 2000 mg/kg bw, with maximal response **[almost 40%, estimated from a graph]** at 24 hours. The microarray analysis patterns showed only "slight" overlap for the response to the 20 and 2000 mg/kg bw DEHP treatments. The genes up-regulated by DEHP 2000 mg/kg bw included genes involved in apoptosis, cell proliferation, metabolism, stress response, cell adhesion, immune response, DNA repair, and expression sequence tags. The RT-PCR results for apoptosis-related genes showed no effect of treatment with DEHP 20 mg/kg bw except for a transient increase in *bcl-2* at 6 hours. Following treatment with DEHP 2000 mg/kg bw, significant increases in message were seen as early as 3 hours for *capsase-2*, *caspase-3*, *caspase-8*, *caspase-9*, *bax*, *fas*, and *fasL*. These increases persisted through the 24-hour time point and, for the caspases, through the 72-hour time point. There was a significant decrease in *bcl-2* message in the 2000 mg/kg bw group at 3, 6, and 24 hours after the dose. The authors concluded that DEHP affected all stages of spermatogenesis with apoptosis peaking at 24 hours. They postulated that the decrease in identification of apoptosis at 72 hours may have represented Sertoli cell phagocytosis of apoptotic spermatogenic cells.

Strengths/Weaknesses: The scientific rigor of the approach and the combined use of histology and focused gene expression methods are strengths. The RT-PCR experiments were not described in adequate detail, and there is an apparent lack of adequate controls. Other weaknesses include lack of replicates and insufficient and inappropriate statistical analyses.

Utility (Adequacy) for CERHR Evaluation Process: This study is adequate for the evaluation process, although it does not fundamentally add to our knowledge of the initiating mechanisms of DEHP damage. These data may help identify the subcellular pathways of cell death used by the testis after DEHP exposure.

Kim et al. *(138)*, supported by the Japanese Ministry of Education, Science, Sports, Culture, and Technology, evaluated DEHP-associated changes in testosterone catabolism in 4-week-old male Wistar rats. Animals (6/group) were given DEHP orally **[purity not specified; gavage assumed]** in corn oil at 0, 100, or 1000 mg/kg bw/day for 5 days. Two weeks later, animals were killed and testes and livers were removed and weighed. The tissues were homogenized, and microsomal fractions were isolated by centrifugation. Microsomal protein was evaluated using SDS-PAGE and Western blot for CYP2C11 and CYP3A2. Blood was obtained for estimation of testosterone using a commercial RIA kit. The microsomal fraction was used for quantification of 17β-hydroxylation of androstenedione, 6β- (CYP3A2) and 16α-hydroxylation (CYP2C11) of testosterone, and 3-hydroxylation (CYP3A2) and N-desmethylation (CYP2C11) of diazepam. HPLC was used to quantify the reaction products. 5α-Reductase was quantified in the microsomal fraction **[method specified by reference to a 1979 article]**. Total RNA was extracted from testes, and RT- PCR was used to quantify aromatase (CYP19). Statistical testing was performed using ANOVA **[post hoc testing was not discussed]**. Results are summarized in Table 26. The authors proposed that the observed increase in 5α-reductase may have been a compensatory response to reduced testicular testosterone, and that the reduction in aromatase would be expected to result in decreased estrogen availability. The alterations in CYP isoforms were characterized by the authors as more sensitive markers of DEHP disruption of testicular steroid catabolism than testosterone synthesis from androstenedione.

Table 26. Testosterone Metabolism after DEHP Treatment of 4 Week-old Rats

	DEHP dose group (mg/kg bw/day × 5 days)	
Parameter	100	1000
Testis weight	↔	↓62%
Serum testosterone	↔	↓34%
Androstenedione 17β-hydroxylation, testis	↔	↔
5α-Reductase, testis[a]	↑21%	↑36%
5α-Reductase, liver	↔	↔
6β-Hydroxylation of testosterone, testis[a]	↑1.2-fold	↑1.3-fold
16α-Hydroxylation of testosterone, testis[a]	↑1.2-fold	↑1.6 fold
3-Hydroxylation of diazepam, testis	↔	↑1.4-fold
N-Desmethylation of diazepam, testis	↔	↑4.6-fold
Microsomal CYP3A2, testis, Western blot	↔	↑2.3-fold
Microsomal CYP2C11, testis, Western blot	↔	↑2.6-fold
Aromatase mRNA expression, testis[b]	↓41%	↓53%

[a]Estimated from graph.
[b]**[Results in text state that aromatase was reduced *by* 59 and 47% compared to control. Figure suggests that the results should have stated that aromatase was reduced *to* 59 and 47% of control values.]**
↑,↓ Statistically significant increase, decrease in the parameter; ↔ no significant change in the parameter.
From Kim et al. *(138)*.

Strengths/Weaknesses: The use of a recovery period after dosing ended complicates the interpretation of DEHP effects. The absence of histopathology renders the condition of the testis at the end of the experiment uncertain. The absence of measurements of the end-products of each of these enzymatic reactions also reduces confidence in the biological importance of the findings. Not having a biological correlate or sequelae to these changes also reduces their value.

Utility (Adequacy) for CERHR Evaluation Process: The study is inadequate for the evaluation process. It adds slightly to our appreciation of testosterone catabolic pathways, although how and why the findings are important, particularly with the two-factored dosing scheme (dosing plus recovery), is not clear.

Kim et al. *(139)*, supported by the Japanese Ministry of Education, Science, Sports, Culture, and Technology, performed a follow-up study to evaluate cytosolic phospholipase A_2 activity in testes obtained from DEHP-treated rats. Four-week-old male Wistar rats were given DEHP **[purity not specified]** in corn oil at 0, 100, or 1000 mg/kg bw/day for 5 days. Testes were removed 2 weeks after the last treatment, and cytosol and microsomes were isolated by centrifugation of S-9 fractions. Phospholipase A_2 activity was measured using a commercial kit. Phospholipase A_2, cyclooxygenase-2, 12-lipoxygenase, and CYP4A1 were ealuated using SDS-PAGE with chemiluminescence detection. Arachidonic acid was measured using reverse-phase HPLC. Statistical analysis was by Dunnett test. DEHP treatment did not alter rat body weight, although serum testosterone and testicular weight were decreased by treatment with DEHP 1000 mg/kg bw/day. Cytosolic phospholipase A_2 was decreased to 62% of control values by DEHP 1000 mg/kg bw/day. Arachidonic acid concentrations were decreased 18% by DEHP 100 mg/kg bw/day and 24% by DEHP 1000 mg/kg bw/day. The expression of lipooxygenase-12 and CYP4A1 was increased by DEHP **[quantification not given]**, but cyclooxygenase-2 was not altered by treatment. The authors postulated that a DEHP-associated decrease in arachidonic acid may be at least partly responsible for a decrease in testosterone synthesis. The decrease in arachidonic acid may be due to a decrease in formation by phospholipase A_2 and a decrease in metabolism by lipooxygenase-12 and CYP4A1. The increase in CYP4A1 may have been due to PPARα activation by DEHP (not measured in this study) and would be expected to increase lipid oxidation. Lipoxygenase activity would also be expected to generate superoxide.

Strengths/Weaknesses: This study used the same confounding dosing-plus-recovery schedule as used in the previous study *(138)*. However, the evaluation of active fatty acids is a significant plus, as it broadens our appreciation of biological sequelae of DEHP exposure. The dosing sequence renders the results difficult to interpret.

Utility (Adequacy) for CERHR Evaluation Process: The dosing and recovery schedule make this study difficult to interpret and prevent it from being useful in the evaluation process. The focus on phospholipase A_2 and arachidonic acid pathways may be a potentially important contribution to mechanistic understanding of DEHP toxicity but is not interpretable at this time based on the treatment schedule.

Akingbemi et al. *(80)*, supported by NIEHS, evaluated the effect of DEHP on Leydig cell function in male Long-Evans rats exposed during pregnancy, lactation, or post-weaning stages (pregnancy and lactation results are presented in Section 3.2). Prepubertal rats were exposed to DEHP (>99% purity) on PND 21–34, 35–48, or 21–48, and young adult rats were exposed on PND 62–89. DEHP was administered by gavage in corn oil at 0, 1, 10, 100, or 200 mg/kg bw/day (n = 10/dose group). Animals were decapitated within 24 hours of the final dose, and trunk blood was collected for measurement of LH and testosterone by RIA. Testes and seminal vesicles were weighed, and testicular interstitial fluid was collected for measurement of fluid testosterone by

RIA. Testicular histology was evaluated. Leydig cell cultures were prepared from testes by Percoll density gradient preceded in 49- and 90-day-old rats by centrifugal elutriation. The resulting Leydig cell preparations were 95–97% pure, as evaluated by staining for 17-hydroxysteroid dehydrogenase. Cultures were evaluated after 3 hours incubation with and without a maximally stimulating concentration of ovine LH. Testosterone was measured in the medium. The activity of different enzymes in the testosterone biosynthesis pathway were evaluated by incubating Leydig cells for 3 hours with saturating concentrations of substrate for the enzyme of interest: 22R-hydroxycholesterol for P450-cholesterol side-chain cleavage enzyme, pregnenolone for 3β-hydroxysteroid dehydrogenase, progesterone for P450-17α hydroxylase/17,20-lyase, and androstenedione for 17β-hydroxysteroid dehydrogenase. The steroid products were measured by HPLC with ultraviolet detection. Statistical analysis was by ANOVA and Duncan multiple range test.

There were no treatment-related effects on body weight gain or feed consumption. Treatment of prepubertal rats on PND 21–34 or PND 35–48 did not produce alterations of serum LH or testosterone, but longer treatment (from PND 21–48) produced a dose-related increase in serum LH and testosterone and in interstitial fluid testosterone that was statistically significant at a DEHP dose level of 10 mg/kg bw/day (Figure 5). Leydig cells isolated from rats after DEHP treatment on PND 21–34 showed a decrease in basal and LH-stimulated testosterone production in vitro (LOAEL 100 mg/kg bw/day). Leydig cells isolated from rats after DEHP treatment on PND 35–48 displayed decreased basal and LH-stimulated testosterone production in vitro (LOAEL 10 mg/kg bw/day) and decreased activities of enzymes in the testosterone synthesis pathway. All tested enzyme activities were affected, with the most sensitive being 17β-hydroxysteroid dehydrogenase (reduced 74% at 10 mg/kg bw/day compared to control; other enzyme activities were significantly reduced at DEHP dose levels of 100 or 200 mg/kg bw/day). Leydig cells isolated from prepubertal rats that had been treated on PND 21–48 showed an increase in basal and LH-stimulated testosterone production with a LOAEL of 10 mg/kg bw/day. There was no effect of DEHP treatment in adults at any tested dose on serum testosterone or LH or on in vitro Leydig cell steroidogenesis **[data not shown]**. There were no effects of any treatment on testicular histology.

The authors postulated that the decrease in in vitro steroidogenesis after treatment of rats from PND 35–48 without a decrease in evidence of in vivo testosterone synthesis may have represented enzyme inhibition that was not great enough to alter in vivo serum testosterone levels; i.e., the animals could compensate for the treatment effect on synthesis. The authors pointed out that the substrates were provided for the Leydig cell culture experiments in higher amounts than may typically be available in vivo. The authors believed that the increased testosterone and LH serum levels and the increase in in vitro testosterone biosynthetic ability of Leydig cells after 28 days of DEHP treatment represented a compensatory mechanism, with a disruption of the negative feedback mechanism that typically maintains homeostatic testosterone levels. They identified 1 mg/kg bw/day as a NOAEL in their experiments and 10 mg/kg bw/day as a LOAEL.
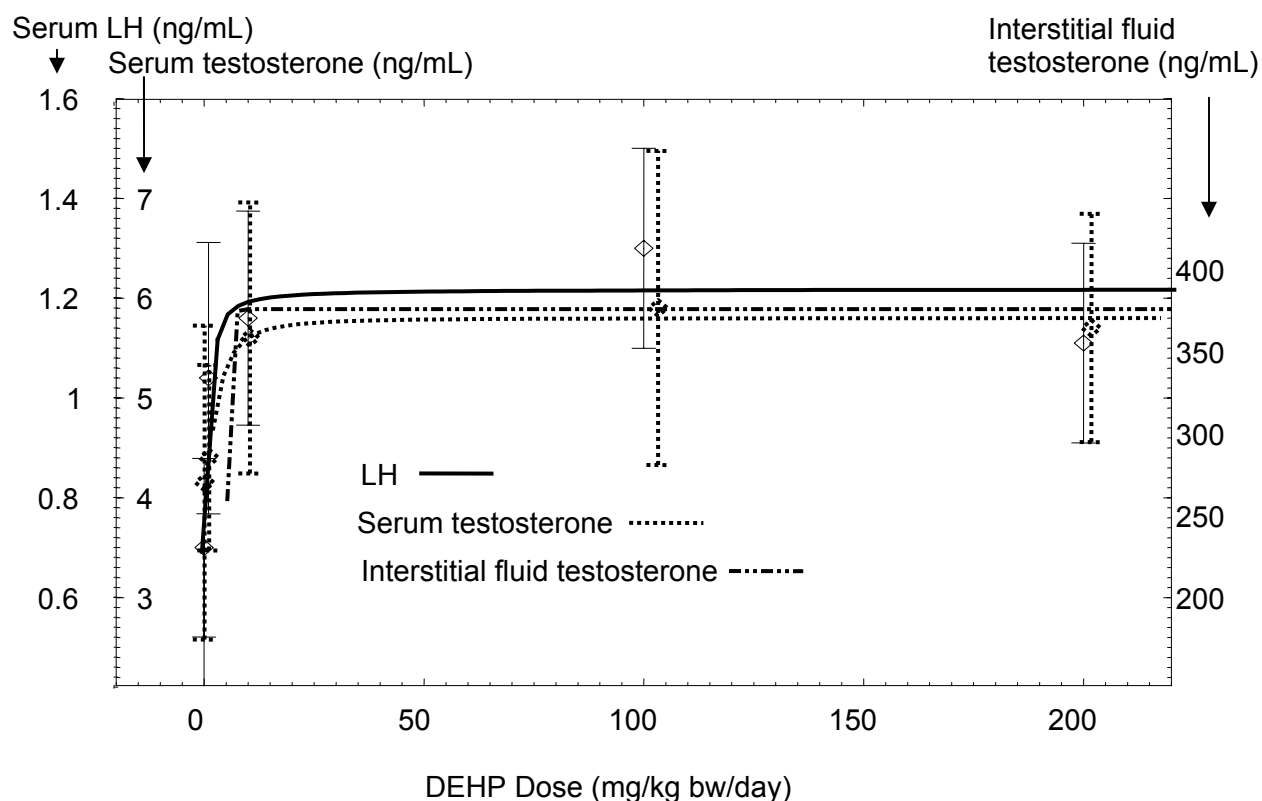
Figure 5. Effect of DEHP Treatment in Male Rats from PND 21–48.
LOAEL = 10 mg/kg bw/day by pair-wise testing (drawn from data presented by Akingbemi et al. *(80)*.

Strengths/Weaknesses: This study benefits from intelligent study design, technical expertise, and appropriate dosing intervals and quantities. Some of the groups have fewer animals than might be optimal. Appropriate statistics is a strength.

Utility (Adequacy) for CERHR Evaluation Process: This study is adequate and useful for the evaluation process because it helps identify a vulnerable window of exposure and during that more-vulnerable period does a good job of parsing the dose-response curve. The study effectively separates inherent effects from compensatory change by the smart use of ex vivo cultures and identifies critical enzymatic steps in steroidogenesis.

Akingbemi et al. *(140)*, supported by NIEHS, evaluated Leydig cell hyperplasia and steroid production in response to DEHP treatment of male Long-Evans rats as a follow-up to the previous study *(80)* in which treatments were given on PND 21–48. Three experiments were described in the current paper, with comparisons performed using ANOVA and post hoc Dunnett multiple range test. In the first experiment, animals were gavaged on PND 21 to PND 90 or to PND 120 with DEHP **[purity not specified]** in corn oil at 0, 10, or 100 mg/kg bw/day (n = 10/group). These treatments caused no significant change in body weight or paired testis weight at 120 days of age. At the end of the treatment periods, serum LH and testosterone were measured by RIA, and Leydig cells were harvested by collagenase digestion and Percoll density centrifugation **[methods obtained from the earlier paper *(80)*.]** The Leydig cells were cultured

for 3 hours with and without maximally stimulating concentrations of ovine LH. Medium testosterone was measured by RIA and normalized to cell number. In the animals treated until PND 90, serum LH and testosterone were increased in both DEHP dose groups compared to controls, and testosterone production in Leydig cell culture was significantly reduced, both with and without added LH. On PND 120, the increase in serum LH and testosterone and the decrease in basal and stimulated testosterone production in culture were identified only in the animals exposed to DEHP 100 mg/kg bw/day. The study authors believed the results were consistent with Leydig cell hyperplasia because serum testosterone was increased in the face of a reduction in testosterone production per Leydig cell.

In the second experiment, the possibility of Leydig cell hyperplasia was evaluated using markers of cell division (PCNA, cyclins D_3 and G_1, and p53, estimated by RT-PCR of steady-state mRNA levels in Leydig cells), incorporation of tritiated thymidine in cultured Leydig cells, and counting of Leydig cells in 10 sections/testis. The treatment regimens were the same as in the first experiment. mRNA levels were increased in both DEHP exposure groups for PCNA and both cyclins and in the high-dose group for p53. Leydig cell numbers were also increased in both DEHP dose groups. **[Data for these outcomes were only described for the animals treated until PND 90.]** Tritiated thymidine incorporation was increased in both DEHP exposure groups in animals treated until PND 120. **[Data not described or shown for animals treated until PND 90.]**

The third experiment evaluated the hypothesis that a DEHP-associated increase in LH induced estrogen synthesis. Male rats were gavaged with DEHP in corn oil at 0, 10, or 100 mg/kg bw/day from PND 21 to PND 48 or 90 at which time serum estradiol was measured by RIA. Leydig cells were cultured for 3 hours with or without exogenous LH, and estradiol in the medium was measured and normalized for cell number. Aromatase mRNA was measured in Leydig cells. Serum estradiol was increased on PND 48 in rats in both DEHP exposure groups. Basal estradiol production in cultured Leydig cells was increased in the 100 mg/kg bw group, and LH-stimulated estradiol production was increased in both DEHP dose groups on PND 48. Aromatase mRNA was also increased in Leydig cells from both DEHP-treated groups. On PND 90, there was no significant difference between control and DEHP-treated animals in serum estradiol level, in basal or LH-stimulated estradiol production in culture, or in aromatase mRNA. MEHP but not DEHP was detectable in serum by HPLC on PND 48 and 90, suggesting that MEHP mediated the testicular effects of DEHP treatment. Serum MEHP was more than 6-fold lower **[estimated from a graph]** on PND 90 than on PND 48.

The authors concluded that DEHP increases Leydig cell populations associated with chronically increased LH and testosterone levels, and that a decrease in testosterone and increase in estradiol synthesis (per cell) was consistent with induction of aromatase activity in Leydig cells. They further proposed that the increase in estradiol and LH could act together to increase cyclin proteins and thereby increase Leydig cell numbers. They concluded that the Leydig cell hyperplasia identified in their study might have implications in the production of testicular neoplasms, and that chronic exposure to DEHP had anti-androgenic and estrogenic activity.

Strengths/Weaknesses: The multiple technical approaches and good design of these studies is buttressed by the sequential hypothesis-testing approach to produce an excellent paper. This report extends our knowledge about the hormonal perturbations to include hyper-estrogenization, and deepens our appreciation of the complexity of this syndrome.

Utility (Adequacy) for CERHR Evaluation Process: The data are adequate for the evaluation process and increase the useable data-set by adding estrogen-induced effects to the spectrum of

sequelae. The use of 100 mg/kg bw/d is not meant to plumb a NOAEL but to employ an effective dose level. This paper suggests that elevated levels of estradiol might be present in some of these animal models and human populations and should be considered in further evaluations.

Ljungvall et al. (141), supported by the Swedish Environmental Protection Agency, treated pre-pubertal male pigs with DEHP (99.6% pure) 50 mg/kg bw/dose, estradiol benzoate 0.25 mg/kg bw/dose, or peanut oil vehicle. Treatments were given twice/week im for 5 weeks, beginning 1 week after weaning, at 6 weeks of age **[DEHP 50 mg/kg bw/dose twice/week would be 100 mg/kg bw/week or about 14 mg/kg bw/day; the Expert Panel notes that this calculated daily dose after twice weekly injection may not be comparable to a similar oral daily dose. The authors justified the im route as the most practical route for treating young pigs.]** Evaluations were planned for 5 days after the last treatment (the immediately-evaluated group) and for 4.5 months after the last treatment period (the delayed-evaluation group). The immediately-evaluated group consisted of 1 male pig from each of 4 litters assigned to each treatment (4 male pigs/treatment group). The delayed-evaluation group consisted of 5 male pigs from 4 litters assigned to DEHP, an additional 5 male pigs from 4 litters assigned to the vehicle control, and 3 male pigs from 3 litters assigned to estradiol benzoate. In the immediately-evaluated animals, blood was sampled weekly during treatment for plasma testosterone, 17β-estradiol, and LH by RIA. Five days after the last treatment, immediately-evaluated animals were necropsied and relative testicular weight was recorded. In the delayed-evaluation group, blood was collected for the plasma testosterone, 17β-estradiol, and LH before the first treatment, after the last treatment, and at the time of slaughter at 7.5 months of age. Necropsy was performed and relative testicular and seminal vesicle weights were recorded. Hematoxylin and eosin-stained testis sections were evaluated by light microscopy, and immunohistochemistry for vimentin was performed. Digitized images of testis sections were evaluated for Leydig cell area and tubule diameter. Leydig cell area relative to tubule area, the proportion of testicular morphologic abnormalities, and the proportion of tubules in Stage VIII were compared between groups with the Wilcoxon-Mann-Whitney test. ANOVA was used for other comparisons.

In the immediately-evaluated group, there were no significant differences in plasma testosterone concentration in DEHP- or estradiol benzoate-treated animals compared to control. At 7.5 months of age, DEHP treatment was associated with an increase in plasma testosterone, and estradiol benzoate treatment was associated with a decrease in plasma testosterone compared to control. There were no DEHP-related effects on plasma estradiol. Plasma LH was described by the authors as tending to be higher after 2 weeks of DEHP treatment ($P = 0.08$) in the immediately-evaluated group. In the delayed-evaluation group, LH was lower in the DEHP group prior to treatment compared to the control group. There were no significant effects of DEHP treatment on relative weight of the testis or seminal vesicles. There were no significant differences in testis morphology in the DEHP-treated animals, although Leydig cell area relative to tubule area was increased by DEHP to 0.45 ± 0.026 (SEM) from a control value of 0.35 ± 0.033. The authors noted a "weak indication that spermatogenesis was affected by DEHP" based on an increase in proportion of Stage VIII tubules ($P = 0.1$). They indicated that although they had characterized DEHP toxicokinetics in male pigs after oral administration (142), they did not have data on metabolism or kinetics after im administration in male pigs. They noted that in rats, parenterally administered DEHP is metabolized to MEHP. The authors concluded that DEHP administered before puberty in male pigs increased plasma testosterone and testosterone-producing cells 4.5 months after the last exposure.

Strengths/Weaknesses: The variety of appropriate measures and appropriate time points and examination of a new species make this a useful and relevant study. The im injection route complicates the toxicokinetic profile somewhat, but does not fundamentally detract from the

utility of this study. The small numbers of animals used, while logistically justifiable, complicates the interpretation, and reduces our confidence in the veracity of the data. The twice-weekly administration, in the absence of blood level measures of MEHP, also weakens the direct comparison of these data to other daily dosed lab animal data.

Utility (Adequacy) for CERHR Evaluation Process: This study is adequate and useful for the evaluation process in showing Leydig cell hypertrophy in young swine (in parallel with that shown in rats in other studies). The serum hormone changes previously documented in rats do not appear as consistently in these swine, perhaps because of the low numbers and twice-weekly dosing schedule. These data are sufficient to show that twice-weekly dosing with 50 mg/kg to young swine can cause Leydig cell hypertrophy 4.5 months after the end of exposure.

4.2.3 Male and Female

Kim et al. (143), support not indicated, evaluated the effects of DEHP (99% pure) on gonad development and serum vitellogenin in Japanese medaka. DEHP was dissolved in acetone and added to aquarium water at up to 250 µL/L; controls received acetone. Serum vitellogenin was assessed after exposure for 5 days to DEHP-containing water at 0, 10, 50, and 100 µg/L, n=10 fish/concentration **[both males and females; ratio not indicated]**. Serum was evaluated using SDS-PAGE, and a band at 200 kDa was taken to represent vitellogenin based on the finding that 17β-estradiol produced a prominent band in this location. The heaviness of the band on the gel was evaluated with the naked eye to compare the effects of treatments on vitellogenin content of serum, with the finding that DEHP exposure appeared to decrease the heaviness of the band in female fish **[the text says that this effect occurred at 1 µg/L, which was not listed in the Methods as 1 of the concentrations used in this experiment]**. A chronic experiment was performed using DEHP concentrations of 0, 1, 10, and 50 µg/L with exposure from 1 or 2 days after hatching until 3 months of age. Serum vitellogenin was evaluated in 1 male and 1 female per exposure level. The remaining fish were fixed in neutral buffered formalin, weighed, and measured; gonads were removed for weighing and histologic evaluation. After the chronic exposure, vitellogenin was again described by visual inspection as reduced in females **[effect level not given]**. Mean weight and length were not altered by treatment. Relative testicular weight and testicular histology were not altered by treatment, but relative ovarian weight was reduced at DEHP concentrations of 10 and 50 µg/L **[to 33 and 38% of control values]**. Histological evaluation revealed impaired maturation of oocytes, with the achievement of yolk deposition in 54% of control females, 37% of females exposed to DEHP 1 mg/L, no females exposed to DEHP 10 µg/L, and in 22% of females exposed to DEHP 50 µg/L. The authors concluded that the decrease in serum vitellogenin and the impairment of oocyte maturation associated with DEHP exposure was consistent with an anti-estrogenic effect of DEHP. **[Actual concentrations of DEHP were noted to be 88% of target at 0 hours and 57% of target at 72 hours. The authors calculated that their protocol of changing the DEHP solutions every 48–72 hours would have resulted in exposure to 73–78% of the nominal concentrations as a time-weighted average.]**

Strengths/Weaknesses: The strengths of this paper include the measurement of DEHP in the aquarium water. The paper is weakened by the fact that vitellogenin was not determined by Western blotting or some other method of positive identification and by the tiny numbers of animals used to collect the vitellogenin data after "chronic" exposure (1/group). A repeated *t*-test was not the correct statistic to use to compare multiple dose groups to controls. Consequently, it is difficult to believe the significance values for the relative gonadal weights, especially given that the mean and SD for the controls are the same approximate size (making a reduction by approximately 67% in the middle-dose group of dubious significance). The bar graph

representation of the oocyte progression data was useful. The non-dose-related changes increase our uncertainty of the veracity and replicability of these findings.

Utility (Adequacy) for CERHR Evaluation Process: This paper is adequate to show that water-based exposure to about 7 and about 35 µg/L DEHP was effective in reducing ovarian development, although the actual degree of this reduction is as difficult to ascertain. The relevance to mammalian reproductive processes of these data is uncertain.

Mitsubishi Chemical Safety Institute, Ltd. (65), in an unpublished report, described a 65-week oral-dose toxicity study of DEHP in marmosets. **[Data from this report also appeared in an abstract (144).]** The study was sponsored by the Japan Plasticizer Industry Association. DEHP (99.6% purity) was administered by gavage in corn oil to juvenile marmosets beginning at 90–115 days of age at dose levels of 0, 100, 500, and 2500 mg/kg bw/day (n = 9 males and 6 females/dose group). The rationale for the age at onset of treatment was to start treatment as early "as technically possible." The treatment period was designed to extend to the age of sexual maturation at about 18 months. Wasting and death occurred in 1–3 males/group but was not treatment related, and these animals were replaced. Blood was drawn every 13 weeks for hematology, chemistry, and determination by RIA of testosterone, estradiol, triiodothyronine, and thyroxine. Animals were killed 1 day after the final DEHP dose. Six males and all 6 females in each dose group underwent gonad removal. The left testis and epididymis were fixed in Bouin solution for light microscopy. The right testis and epididymis were frozen for sperm counting and a portion of the frozen right testis was used for determination of zinc, sorbitol dehydrogenase, γ-glutamyl transpeptidase, total glutathione, glutathione-S-transferase, and glutathione peroxidase. Left ovaries were frozen for histochemistry for 3β-hydroxysteroid dehydrogenase. Livers from both sexes were analyzed for enzyme levels or activities. An additional 3 males per dose group were perfused with glutaraldehyde, and testes were used for 3β-hydroxysteroid dehydrogenase histochemistry and for electron microscopy. Data were analyzed using ANOVA with post hoc Dunnett or Scheffé test or Kruskal-Wallis test with post hoc Dunnett rank-sum or Scheffé test.

There were no treatment-related alterations in hematology, blood chemistry, or blood hormones. Body weights were not affected by treatment. Organ weights were not affected except for ovarian and uterine weights, which were significantly increased at DEHP dose levels of 500 and (for ovaries) 2500 mg/kg bw/day (Table 27). Testicular enzymes were not altered by treatment, although there was a 16–21% decrease in testicular zinc at the 100 and 500 mg/kg bw/day dose levels. There were no alterations in sperm counts; however, 1 animal in each of the DEHP groups was omitted from the analysis as having exceptional values due to these animals being diagnosed as "growing." Hepatic cytochrome P450 content and testosterone 6β-hydroxylation were significantly increased in females at DEHP 500 mg/kg bw/day, but hepatic peroxidase enzymes were not altered by treatment. There were no gonadal histopathologic findings by light or electron microscopy that were attributable to DEHP treatment, although small growing animals had testicular findings consistent with immaturity. Degenerative testis changes similar to those described elsewhere for rodents were not identified in marmosets. Immunohistochemistry findings were not altered by treatment. The authors concluded that in spite of demonstration of absorption of the compound, as manifested by adaptive hepatic changes, DEHP in marmosets did not exert testicular toxicity at a dose level as high as 2500 mg/kg bw/day. The authors suggested, based on an accompanying pharmacokinetic study (reviewed in Section 2.1), that the most likely explanation for the lack of testicular toxicity of DEHP in marmosets as opposed to rodents was the limited accumulation of DEHP and its metabolites in the marmoset testis.

Strengths/Weaknesses: Strengths of this study are the use of a primate species, the length of exposure, the determination of DEHP and MEHP levels in numerous tissues, and the numerous end points relevant to the reproductive system.

Utility (Adequacy) for CERHR Evaluation Process: These data are useful for the evaluation process and are sufficient to show that marmoset exposure to DEHP starting at about 3 months of age and lasting until about 18 months of age (sexual maturity) had no discernable effect on the testis, either at the ultrastructural, histochemical, or gross functional (sperm count) level. The data are adequate to show an effect on ovary and uterine weight in these young adults at necropsy at administered doses of 500 and (for ovary) 2500 mg/kg bw/day.

Table 27. Ovary and Uterine Weight Findings in a Marmoset 65-Week DEHP Feeding Study

Organ weight[b]	Dose level (mg/kg bw/day)			Benchmark dose[a] (mg/kg bw/day)			
	100	500	2500	BMD_{10}	$BMDL_{10}$	$BMD_{1\,SD}$	$BMDL_{1\,SD}$
Ovary, absolute	100	180*	169*	507	259	2063	1196
relative	106	167*	162*	572	303	1999	1173
Uterine, absolute	106	188*	168	562	258	2545	1356
relative	100	167*	150	677	296	2759	1374

[a]Calculated by CERHR. See Table 20 for definitions and a discussion of the use of benchmark dose in this report.
[b]Data presented as percent of control. *Significantly different from control, $P < 0.05$. From Mitsubishi Chemical Safety Institute, Ltd. report (65)).

Schilling et al. *(113)*, sponsored by the European Council for Plasticizers and Intermediates (a sector group of CEFIC, the European Chemical Industry Council), performed a 2-generation reproductive toxicity study of DEHP in Wistar rats. DEHP (99.7% pure) was administered in feed at 0, 1000, 3000, and 9000 ppm, resulting in estimated DEHP intakes of 0, 113, 340, and 1088 mg/kg bw/day. F_0 male and female animals were 36–38 days old when they were placed on test (n = 25/group). Animals were mated at least 73 days after the beginning of treatment. Females littered and raised their own pups. On PND 4, litters were standardized to 8 (4 males and 4 females where possible). Pups were weaned on PND 21. Treated feed was provided throughout the gestation and lactation period, and pups were weaned to the diet assigned to their parents. During the rearing period, 25 males and 25 females per dose group (1/sex/litter where possible) were assigned to be F_1 parents. Mating (non-sibling) occurred at least 75 days after assignment. F_2 litters were standardized on PND 4 and weaned on PND 21. On PND 28, 10 F_2 offspring/sex/dose group were evaluated by functional observation battery and motor activity testing and an additional 10 F_2 offspring/sex/dose group were evaluated in a water maze test of learning and memory. Estrous cycle was monitored daily for all F_0 and F_1 females for at least 3 weeks prior to mating. Cauda epididymal sperm were assessed for motility, morphology, and head count in F_0 and F_1 males at necropsy. Sperm head count was also performed in testes. Reproducing females were killed after weaning, and uteri were stained in 10% ammonium sulfide for calculation of implantation sites, used in the calculation of postimplantation loss. Male pups were evaluated on PND 12 and 13 for the presence of nipples and areolae. Age and weight at vaginal opening and preputial separation were assessed in pups. One pup/sex/litter was killed on or after PND 21, and brain, spleen, thymus, liver, kidneys, testes, ovaries, and uterus (with oviducts and cervix) were weighed. Gross necropsy was performed on pups culled on PND 4 and on those pups not selected as parents for the next generation or for neurobehavioral testing (on PND 21). Histologic examination was performed on reproductive organs and other selected organs, and follicle counts were performed in ovaries. Statistical comparisons were made using

the Dunnett test for means and the Fisher exact test for proportions or the Kruskal-Wallis test with Wilcoxon test for proportions of affected pups/litter, pup organ weights, and several neurobehavioral endpoints.

Selected results are given in Table 28. In general, the high-dose level (9000 ppm) was associated with a decrease in feed consumption and weight gain at several intervals during the study. Clinical signs leading to unscheduled sacrifice were present in 1 high-dose F_0 female. Six high-dose adult F_1 females died or were killed in moribund condition during the pregnancy or lactation periods; 3 of these females were found to have liver necrosis. F_2 pups in the high-dose group were smaller and gained less weight from birth through the period of assessment of functional observation battery and water maze testing. Grip strength was reduced in males, and hind-limb splay was reduced in both sexes in the high-dose group. There were no other treatment-related findings in the functional observation battery or in the water maze. Differential ovarian follicle counts of F_0 and F_1 adults showed a deficit in growing follicles and corpora lutea in the high-dose group **[expressed per follicle]**.

The authors concluded that reproductive performance and fertility were affected at the 9000 ppm dose level with a NOAEL of 3000 ppm, and that developmental toxicity was noted at 3000 and 9000 ppm, with an increase in stillbirth, an increase in PND 0–4 pup mortality, retardation of F_2 pup body weight gain, reduced male anogenital distance, and increased retained nipples/areolae in males. A delay in sexual maturation was also noted in F_1 male and female offspring at 9000 ppm. The NOAEL for developmental toxicity was considered by the study authors to be 1000 ppm. The alterations in pup organ weights were noted, but the changes in spleen and thymus weight were assessed as causally related to the body weight alterations and not as primary effects of DEHP. The alterations in liver weight were considered likely to be due to peroxisome proliferation and were not representative of developmental toxicity. The NOAEL for systemic toxicity was considered by the study authors to be 1000 ppm. **[The lowest BMD_{10} for reproductive toxicity was 2325 ppm ($BMDL_{10}$ 2045 ppm) for F_2 pup survival on PND 0–4. Based on the dose regression provided by the authors, the corresponding DEHP intake is 263 mg/kg bw/day ($BMDL_{10}$ 231 mg/kg bw/day).]**

Strengths/Weaknesses: A strength of this study is the completeness and widely accepted rigor (i.e., to GLP standards) with which it was performed, as well as the number of animals and the number of endpoints.

Utility (Adequacy) for CERHR Evaluation Process: This study is useful for the evaluation process and shows a NOAEL for developmental toxicity at 1000 ppm based on F_1 and F_2 pup survival, effects on reproductive development, and pup postnatal growth and organ weight effects, a NOAEL for reproductive toxicity at 3000 ppm based on fertility and organ weights, and a NOAEL for general effects below 1000 ppm based on pup liver weight increases.

Table 28. Results of 2-Generation Test of DEHP in Wistar Rats

Parameter		Dose in feed (ppm)			Benchmark dose (ppm)[a]			
		1000	3000	9000	BMD_{10}	$BMDL_{10}$	$BMD_{1\,SD}$	$BMDL_{1\,SD}$
F_0 Feed consumption during pregnancy		↔	↔	↔				
F_0 Feed consumption lactation day 1–4		↔	↔	↓18%	5433	4016	7244	5257
F_0 Feed consumption lactation day 4–7		↔	↔	↓21%	6886	5015	8578	5792
F_0 Feed consumption lactation day 7–14		↔	↔	↓33%	6928	4465	6986	4577
F_0 Body weight gain during pregnancy		↔	↔	↓11%	8849	7167	8965	7894
F_0 Body weight on lactation day 21		↔	↔	↓14%	8741	7225	8547	6170
F_0 Males with confirmed mating		↔	↔	↔				
F_0 Males with confirmed fertility		↔	↔	"↓"12%[b]				
F_0 Sperm parameters		↔	↔	↔				
F_0 Females with stillborn pups		↔	↔	↑4-fold	6414	250		
F_0 Litter size (F_1 pups/litter)		↔	↔	↔				
F_1 Pups surviving PND 0–4 (pup basis)		↔	↓4%	↓7%	11,399	8541		
F_1 Pups surviving PND 4–21 (pup basis)		↔	↔	↔				
Live F_1 pups/litter on PND 4, 7, 14, 21		↔	↔	↔				
Sex ratio F_1 pups		↔	↔	↔				
Postimplantation loss per F_0 female		↔	↔	↑2.1-fold	7850	813	9070	7659
F_1 Pup body weight, male	PND 1	↔	↔	↓6%	9274	9034	9139	8389
	PND 4	↔	↔	↔				
	PND 7	↔	↔	↓6%	8780	5470	8919	6431
	PND 14	↔	↔	↓26%	5448	3903	5516	3463
	PND 21	↔	↔	↓31%	4661	3005	4615	2875
F_1 Pup body weight, female	PND 1	↔	↔	↔				
	PND 4	↔	↔	↔				
	PND 7	↔	↔	↓16%	7643	4684	7777	5202
	PND 14	↔	↔	↓27%	5460	3818	5224	3451
	PND 21	↔	↔	↓31%	4733	3386	4583	3084
F_1 PND 1 Anogenital distance, male		↔	↔	↓14%	6943	5417	5514	4242
F_1 PND 1 Anogenital distance, female		↔	↔	↔				
F_1 Males with nipples/areolae per litter		↔	↔	↑38-fold	6238	2222		

Parameter	Dose in feed (ppm)			Benchmark dose (ppm)[a]			
	1000	3000	9000	BMD_{10}	$BMDL_{10}$	$BMD_{1\,SD}$	$BMDL_{1\,SD}$
F_1 Days to vaginal opening	↔	↔	↑12%	7921	6534	5407	3631
F_1 Days to preputial separation	↔	↔	↑19%[c]	5780	4325	3986	2592
F_1 PND 21 Absolute organ weights (sexes combined; male and female changes were similar)							
Brain	↔	↔	↓7%	10,964	9118	6484	4136
Thymus	↔	↓12%	↓39%	2506	2056	3443	2728
Spleen	↓15%	↓13%	↓57%	2446	1265	3962	2578
Liver	↔	↑17%	↔	1271	805	2713	1817
Kidney	↔	↔	↓33%	5946	4458	8487	5214
Testis	↔	↔	↓37%	5357	2995	6122	3863
Ovary	↔	↔	↓32%	8472	2265	9013	6273
Uterus	↔	↔	↓22%	8537	2859	9235	8276
F_1 PND 21 Relative organ weights (sexes combined; male and female changes were similar)							
Brain	↔	↔	↑38%	5292	3104	5274	3095
Thymus	↔	↔	↓12%	2467	1541	9534	9379
Spleen	↓11%	↓8%	↓38%	3450	2224	4300	2801
Liver	↑8%	↑22%	↑30%	1138	911	1184	956
Kidney	↔	↑5%	↔				
Testis	↔	↔	↔				
Ovary	↔	↔	↔				
Uterus	↔	↔	↔				
F_1 Feed consumption GD 0–7	↔	↑5%	↓7%	9107	8803	8893	7787
F_1 Feed consumption GD 7–14	↔	↔	↓9%	8989	8218	8782	7034
F_1 Feed consumption GD 14–20	↔	↔	↔				
F_1 Feed consumption lactation day 1–4	↔	↔	↓32%	4587	3079	6266	4328
F_1 Feed consumption lactation day 4–7	↔	↔	↓31%	4540	2715	5159	3040
F_1 Feed consumption lactation day 7–14	↔	↓12%	↓44%	2556	1848	3352	2193
F_1 Body weight gain during pregnancy	↔	↔	↓15%	6015	3920	7748	5283
F_1 Body weight on lactation day 21	↔	↔	↓21%	8539	5480	8501	5238
F_1 Males with confirmed mating	↔	↔	↔				
F_1 Males with confirmed fertility	↔	↔	"↓"24%[b]				
F_1 Sperm count (testis, epididymis)	↔	↔	↔				

Parameter	Dose in feed (ppm)			Benchmark dose (ppm)[a]			
	1000	3000	9000	BMD_{10}	$BMDL_{10}$	$BMD_{1\,SD}$	$BMDL_{1\,SD}$
F_1 Percent abnormal sperm	↔	↔	↑27%	3061	1174	25,588	9256
F_1 Percent motile sperm	↓2%	↔	↔				
F_1 Females with stillborn pups	↔	↑3-fold	"↑"2.8-fold				
F_1 Litter size (F_2 pups/litter)	↔	↔	↓19%	5790	3137	9657	6697
F_2 Pups surviving PND 0–4 (pup basis)	↔	↓15%	↓13%	2325	2045		
F_2 Pups surviving PND 4–21 (pup basis)	↔	↔	↔				
Live F_2 pups/litter on PND 4, 7, 14, 21	↔	↔	↔				
Sex ratio F_2 pups	↔	↔	↔				
Postimplantation loss per F_1 female	↔	↔	↔				
F_2 Pup body weight, male PND 1	↔	↔	↔				
PND 4	↔	↔	↔				
PND 7	↔	↔	↓11%	7703	2940	9299	6072
PND 14	↔	↔	↓29%	4204	2940	3827	2521
PND 21	↔	↔	↓35%	4085	2790	4142	2787
F_2 Pup body weight, female PND 1	↔	↔	↔				
PND 4	↔	↔	↔				
PND 7	↔	↔	↓11%	7894	5078	10,689	6703
PND 14	↔	↓8%	↓21%	3691	2724	3356	2476
PND 21	↔	↔	↓33%	4523	3094	3652	2318
F_2 PND 1 Anogenital distance, male	↔	↓9%	↓10%	8810	6204	6597	3981
F_2 PND 1 Anogenital distance, female	↔	↔	↔				
F_2 Males with nipples/areolae per litter	↔	↑45-fold	↑54-fold	1610	failed[d]		
F_2 PND 21 Absolute organ weights (sexes combined; male and female changes were similar)							
Brain	↔	↔	↓7%	11,077	9201	5432	3686
Thymus	↔	↔	↓39%	2872	1957	4185	2963
Spleen	↔	↔	↓53%	4059	2262	5555	3828
Liver	↑14%	↑20%	↔	↓1618	1096	2557	1480
Kidney	↔	↔	↓30%	6134	4155	6591	4700
Testis	↔	↔	↓38%	4063	2447	4905	3252
Ovary	↔	↔	↓26%	3157	2015	9484	6354
Uterus	↔	↔	↔				

Parameter	Dose in feed (ppm)			Benchmark dose (ppm)[a]			
	1000	3000	9000	BMD_{10}	$BMDL_{10}$	$BMD_{1\,SD}$	$BMDL_{1\,SD}$
F_2 PND 21 Relative organ weights (sexes combined; male and female changes were similar)							
Brain	↔	↔	↑39%	4598	3379	4307	3000
Thymus	↔	↔	↓12%	7302	4633	9860	6359
Spleen	↔	↔	↓32%	5423	3424	6230	4311
Liver	↑12%	↑24%	↑33%	939	636	1637	1149
Kidney	↑6%	↑6%	↔				
Testis	↔	↔	↓10%	9393	6427	8853	5832
Ovary	↔	↔	↔				
Uterus	↔	↔	↑28%	8469	3304	8913	6381

From Schilling et al. *(113)*.

↑,↓,↔ Increase, decrease, or no change in parameter compared to 0 ppm group by statistical testing. "↓","↑" refers to study author conclusion of a difference in that absence of statistical confirmation.

[a] Calculated by CERHR. See Table 20 for definitions and a discussion of the use of benchmark dose in this report. Benchmark dose was calculated only when a treatment effect was demonstrated by the study authors' analysis.

[b] Histologic changes in testes and/or epididymal sperm led the study authors to conclude that there was a treatment-related reduction in fertility in this dose group although statistical significance was not shown.

[c] Two males with hypospadias, penile hypoplasia, and cleft prepuce.

[d] BMDL calculation could not be performed by EPA program using Hill model, which gave the best fit.

Tanaka et al. *(99)*, support not indicated, gave DEHP (>97% purity) to CD-1 mice in the diet from 5 weeks of age in the F_0 generation to 9 weeks of age in the F_1 generation. A single dietary dose level of 0.03% was used, with control animals receiving untreated basal feed (n = 20/sex/treatment group). At 9 weeks of age, 10 DEHP-treated females were paired with DEHP-treated males, 10 DEHP-treated females were paired with control males, 10 control females were paired with DEHP-treated males, and 10 control females were paired with control males. The females' diet was available to males during the 5-day cohabitation phase. Females reared their own unadjusted litters, which were weaned at 4 weeks of age. One female and male from each litter were retained and fed their dam's diet until 9 weeks of age. Statistical analyses were performed using ANOVA or Kruskal-Wallis test, followed by Bonferroni multiple comparison test. Proportions were evaluated using chi-squared or Fisher test. Based on measured feed consumption, mean DEHP intake by treated males **[rounded by CERHR]** was 46 mg/kg bw/day. Treated females received 53–57 mg/kg bw/day during the preconception period, ~43 mg/kg bw/day during mating, 46–49 mg/kg bw/day during gestation, and 154–171 mg/kg bw/day during lactation. DEHP had no effect on feed consumption or dam body weight. There were no significant treatment effects on the number of pregnant females, number of litters, number of offspring, average litter size or weight, or sex ratio. The authors concluded that, "DEHP caused few significant adverse effects on reproductive or neurobehavioral parameters." **[Neurobehavioral testing of the F_1 offspring, the focus of the study, was discussed in Section 3.2.2.]**

Strengths/Weaknesses: The crossover design of this study is a strength, as are the statistical evaluations and the presentation of the data. The use of a single dose level weakens the study for use in assessing reproductive risk, which admittedly was not the major focus of the study.

Utility (Adequacy) for CERHR Evaluation Process: The data are sufficient to conclude that DEHP dosing between 46 and 154 mg /kg bw/day was insufficient to materially change any reproductive parameter measured in this study in mice.

The National Toxicology Program *(145)* sponsored a multigeneration continuous breeding study in rats with the intent of evaluating whether responses seen at very low doses might be different from, or forerunners of, responses seen at higher doses.. Task 1 of the study was conducted to determine the doses used in subsequent tasks. Sprague-Dawley rats (13-weeks-old; 8/sex/group) were given feed containing 0, 5000, or 10,000 ppm DEHP from 7 days prior to mating and during the cohabitation period, which extended until necropsy. **[The time period of cohabitation was not specified.]** DEHP intake was estimated by the study authors at 0, 321.42, and 643.95 mg/kg bw/day. In litters born during the cohabitation period, anogenital distance was measured on PND 1, and growth and mortality were monitored through PND 21. Signs of systemic toxicity included decreased feed and water intake during lactation in females from the 5000 and 10,000 ppm groups and a decrease in body weight gain in females from the 10,000 ppm group. Reproductive effects included a decrease in uterus, cervix, and vagina weights in PND 21 pups from the 5000 and 10,000 ppm groups. At 10,000 ppm, ratio of anogenital distance to pup weight was increased in female pups and pup weights were decreased on PND 4 and 21.

In task 2 of the study, 17 Sprague-Dawley rats/sex/group were randomly assigned to groups and fed diets containing 1.5 (control group exposed to background DEHP levels in feed), 10, 30, 100, 300, 1000, or 7500 ppm DEHP (99.8% pure) from the first day of the study until the day of necropsy. Due to a lack of reproductive effects in the first litter produced, the study was repeated with 2 additional doses, 1.5 (control) and 10,000 ppm. **[It is not clear why the authors concluded there were no reproductive effects in the 7500 ppm group when several significant effects were observed in the first litter, as discussed below.]** Ranges of DEHP

intake in the F_0, F_1, and F_2 animals were estimated at 0.09–0.12, 0.47–0.78, 1.4–2.4, 4.8–7.9, 14–23, 46–77, 392–592, and 543–775 mg/kg bw/day. At about 5 weeks of age, F_0 rats were fed the DEHP-containing diets for 6 weeks prior to mating and were then cohabitated for 9 weeks. Concentrations of dosing solutions were verified. The first 2 litters delivered during the cohabitation period (F_{1a} and F_{1b}) were counted, weighed, assessed for anogenital distance, and then discarded. The third litter (F_{1c}) was raised by the dam until weaning on PND 21 **[designation for day of birth not specified]**. Following weaning of pups, vaginal cytology was monitored in F_0 females for 14 days. After completion of crossover studies described below, at least 10 F_0 rats sex/group were necropsied. Sperm analyses were conducted, and organs were collected for histopathological evaluation. Ovaries were preserved in Bouin solution. Testes and epididymides were preserved in 2% paraformaldehyde/3% glutaraldehyde. F_1 pups were counted, weighed, and examined for anogenital distance and nipple retention during the lactation period. On PND 16, 1 female per litter was evaluated for vaginal opening, and a second was selected for F_1 mating. One male per litter was selected for mating, and 4 or 5 males per litter were evaluated for testicular descent and preputial separation; both groups of rats were necropsied. At weaning (PND 21), pups were given diets containing the same DEHP concentrations as their parents. On PND 81, the F_1 rats chosen for mating (17/sex/group) were randomly assigned to breeding pairs (preferably non-sibling) and cohabited for 9 weeks. The study conducted in F_0 parents and F_1 offspring was repeated in F_1 parents and F_2 offspring, except that the third F_3 litter born (F_{3c}) did not undergo the continuous-breeding protocol. Selected F_{3c} males were necropsied on PND 63–64 and selected females on PND 60–74. Statistical analyses included Jonckheere test to determine if data should be analyzed by Shirley or Dunn test. Shirley test was used to evaluate data that consistently increased or decreased according to dose. Dunn test was used to evaluate data with severe departures from monotonicity. Additional statistical analyses included Wilcoxon, Cochran-Armitage, and chi-squared tests.

Some systemic effects were consistent across all generations. During numerous time periods of the study and especially at necropsy, body weight gains were decreased in rats from the 7500 and 10,000 ppm groups. Dam body weights during delivery and lactation were decreased by 8–20% in the F_0 10,000 ppm group. Increases and decreases in feed intake were observed at most dose levels. In the F_0 7500 and 10,000 ppm groups, feed intake was decreased during lactation. The liver was identified as a target of toxicity, with increases in liver weight and hepatocellular hypertrophy observed at dose levels ≥1000 ppm. Changes in organ weights and lesions were also observed in kidney at ≥7500 ppm and adrenal gland at 10,000 ppm.

Reproductive toxicity findings in all generations of rats are summarized in Table 29. In the F_0 generation, there were no effects on fertility or estrous cycles. The only reproductive effects observed in the F_0 parents occurred at 10,000 ppm and included decreases in sperm counts and velocity, reductions in testis and epididymis weights, and increased numbers of rats with small testes. Histopathological findings associated with small testes included minimal-to-marked atrophy of seminiferous tubules characterized by loss of germ cells. The lowest dose level producing dose-related effects in F_1 offspring was 7500 ppm, and those effects included decreases in number of live pups/litter, reduced male anogenital distance, and delays in vaginal opening, preputial separation, and age of testicular descent. Additional effects noted in the F_1 offspring from the 10,000 ppm group included decreased live pup weight at birth and during the lactation period and increased ratio of female anogenital distance to body weight.

In contrast to findings in the F_0 generation, fertility was compromised in the F_1 rats from the 10,000 ppm group, which did not produce any viable litters. Other reproductive effects observed in F_1 parents were similar to those observed in F_0 parents but usually occurred at lower dose levels. In the non-mating F_1 adult males of the 300 ppm group, there was a small increase in the

number of animals (2 or 3 of 45) with small testes, epididymides, and seminal vesicles. The effects were not observed at the next higher dose (1000 ppm), but small testes were observed in 2 of 10 males of the 7500 ppm non-mating group. Small testes and epididymides were observed in 21 of 21 animals of the 10,000 ppm non-mating group. A small percentage (3–7%) of non-mating rats treated with ≥1000 ppm had small ventral prostates. In rats that were mated, the only decreases in reproductive organ size occurred in testes at 7500 and 10,000 ppm (8 of 10 and 10 of 10 affected at each dose) and epididymides at 7500 ppm (2 of 10). Histopathological findings observed in all animals of the 7500 and 10,000 ppm groups were consistent with those observed in the F_0 generation and included minimal-to-marked seminiferous tubule atrophy and occasional sperm release failure. Minimal seminiferous tubule atrophy was observed in 1 of 10 males in the 100 and 300 ppm groups. Reductions in numerous reproductive organ weights were observed in mating and non-mating F_1 males treated with ≥7500 ppm. Additional reproductive effects observed in F_1 rats were reduced sperm counts at 7500 ppm and higher and increased uterus and ovary weights at 10,000 ppm. Estrous cycle length was slightly increased at 10,000 ppm. In the F_2 pups, delays in preputial separation and testicular descent occurred at every dose level above the control. **[In no other generation did delays in preputial separation and testicular descent occur at such low doses, but the study authors did not offer any explanations for this observation. The Expert Panel believes these findings are consistent with a problem with the control group in that generation.]** All other effects occurred in F_2 pups of the 7500 ppm group and included delayed vaginal opening and reductions in live pup weight at birth and during the lactation period, male anogenital distance, and survival during the lactation period.

No F_2 adult rats from the 10,000 ppm group were available due to complete infertility in F_1 rats of that group. In F_2 adult rats, a decreased number of cycling females observed in the 300 ppm group was not observed at any other dose level. In non-mating males, rats with small testes and epididymides were observed at ≥300 ppm (1/21), 1000 ppm (1/25), and 7500 ppm (7–11/20). However, in males that were mated, small epididymides and testes were only observed at the 7500 ppm dose level (8/10). Seminiferous tubule atrophy was observed in 10/10 males of the 7500 ppm group. All other reproductive effects in F_2 adults occurred at 7500 ppm and included decreases in pregnancy index, the number of litters per pair, male reproductive organ weights, sperm counts, and sperm motility. In F_3 pups, a decrease in postnatal survival of females was observed only on PND 7 in the 300 ppm group but was not observed on any other day or dose level. All other effects in F_3 pups occurred at 7500 ppm and included decreases in male anogenital distance delayed vaginal opening, preputial separation, and testicular descent, and an increase in male pups with nipples. F_3 pups were the only generation of rats to experience an increase in males with nipples. At necropsy of adult F_3 rats, effects were only observed at the 7500 ppm dose level and included reduced sperm counts and weights of dorsolateral prostate, testis, and epididymis.

The study authors discussed the relevancy of small male reproductive organ sizes observed in both F_1 and F_2 rats of the 300 ppm groups. They noted that although incidences were low, the effects were consistent with phthalate-induced toxicity. The incidence of small testes and epididymides exceeded historical control data from the laboratory. Therefore, the study authors considered the effects as potentially treatment-related. However, the study authors concluded that the overall significance of the effects could not be determined due to lack of histopathological data and lack of adverse reproductive effects at 300 and 1000 ppm.

Two crossover breeding studies were conducted. The first was conducted to investigate the lack of litters in F_1 rats of the 10,000 ppm group. The second was conducted to investigate the decrease in F_3 pup body weight in the 7500 ppm group. The studies were conducted by mating the control and high-dose rats of each sex (n = 17/sex/group) with naïve animals for 7 days or

until a vaginal plug was detected. Pups were counted, weighed, assessed for anogenital distance, and then discarded. Implantation sites were examined in naïve females. The crossover studies (Table 30) demonstrated that pregnancy and fertility indices were reduced in males of the 7500 ppm and 10,000 ppm groups. Implantation sites were reduced in the naïve rats mated with DEHP-treated males. A decrease in pup weight and male anogenital distance was seen in offspring born to females treated with 7500 and 10,000 ppm DEHP and mated to naïve males.

The study authors concluded, "The findings obtained in this study indicate that DEHP is clearly a reproductive and developmental toxicant at 7500 and 10,000 ppm based upon changes in fertility and pregnancy indices, litter data, sperm parameters, sexual development, and/or histopathological changes in testes." Intake at 7500 ppm was estimated at 392–592 mg/kg bw/day, and intake at 10,000 ppm was estimated at 543–775 mg/kg bw/day. **[The lowest BMD_{10} was 787 ppm based on F_3 sperm/cauda. Extrapolating from the authors' estimates of intakes at 1000 mg/kg bw/day, this intake level is 36–61 mg/kg bw/day. The lowest $BMD_{1\ SD}$ is 554 ppm based on F_2 percent motile sperm. Extrapolating from the authors' estimates of intakes at 300 ppm, this intake level is 9–15 mg/kg bw/day. The Expert Panel notes that Sertoli cell vacuolation, which was the endpoint driving the LOAEL in the original CERHR Expert Panel evaluation of DEHP, was not increased by DEHP treatment in this study.]**

Strengths/Weaknesses: Clearly, a major strength of this study is the number of dose evaluated. The relatively small group sizes were compensated by the unusually large numbers of groups and the very low doses used. An additional strength is the fact that more offspring were evaluated early for alterations in the development of the reproductive system; a weakness might be that not all animals were so evaluated. The quality of the histology is another strength. The lack of vacuoles is perplexing, but not lethal to the study.

Utility (Adequacy) for CERHR Evaluation Process: These data are adequate for the evaluation process and show that 10,000 and 7500 ppm are clearly toxic to the developing reproductive system in rats. The Expert Panel considers 300 ppm and 1000 ppm to represent the tail of the dose-response curve in this study, based on the incidence of testicular abnormalities, which would put the NOAEL for these developmental effects at 100 ppm, down in the 3–5 mg/kg bw/day range.

Table 29. Results of Continuous Breeding Multigeneration Test of DEHP in Sprague-Dawley Rats

Parameter	Dose in feed (ppm)							Benchmark dose (ppm)[a]			
	10	30	100	300	1000	7500	10,000	BMD_{10}	$BMDL_{10}$	$BMD_{1\,SD}$	$BMDL_{1\,SD}$
F_0 Females											
Cumulative days to deliver	↔	↔	↔	↔	↔	↔	↑ litter 1				
Estrous cycle effects	↔	↔	↔	↔	↔	↔	↔				
F_0 Males											
Spermatid/testis	↑16.5%	↔	↔	↔	↔	↔	↓31%				
Sperm velocity	↔	↔	↔	↔	↔	↔	↓11%				
Body weight at necropsy	↔	↔	↔	↔	↔	↔	↓6%				
Absolute organ weight											
Cauda epididymis	↔	↔	↔	↔	↔	↔	↓19%				
Epididymis	↔	↔	↔	↔	↔	↔	↓16%				
Testis	↔	↔	↔	↔	↔	↔	↓23%				
No. with small right testis	0	0	0	0	0	0	2/10				
F_0 Matings											
Live pups/litter (F_{1a})	↔	↔	↔	↔	↔	↓ 18%	↓ 21%	7008	3237	6771	2849
Live males/litter (F_{1a})	↔	↔	↔	↔	↔	↓20%	↓ 26%	3802	3303	2650	2227
Proportion liveborn pups	↔	↔	↔	↔	↔	↔	↓4%				
Pup body weight	↔	↔	↔	↔	↔	↔	↓ 9%				
Adjusted for litter size	↔	↔	↔	↔	↔	↔	↓ 10%				
Pup anogenital distance											
Male F_{1a}	↔	↔	↔	↔	↔	↓7%	↓ 14%	10,566	7631	3411	2382
Male F_{1b}	↔	↔	↔	↔	↔	↓8%	↓ 15%	8408	7465	2874	2127
Female, relative to bw	↔	↔	↔	↔	↔	↔	↑10–17%				
Male pup retained nipples	↔	↔	↔	↔	↔	↔	↔				
Pup weights PND 1–21											
male	↔	↔	↔	↔	↔	↔	↓30%				
female	↔	↔	↔	↔	↔	↔	↓30%				
Pup survival, PND 4–21	↔	↔	↔	↔	↔	↔	↔				

F_1 Males and females

Parameter	10	30	100	300	1000	7500	10,000	BMD_{10}	$BMDL_{10}$	$BMD_{1\,SD}$	$BMDL_{1\,SD}$
	Dose in feed (ppm)							Benchmark dose (ppm)[a]			
Age at vaginal opening	↔	↔	↔	↔	↔	↑3 days	↑8 days				
Age at preputial separation	↔	↔	↔	↔	↔	↑4 days	↑11 days				
Age at testes descent	↔	↔	↔	↔	↔	↑3 days	↑6 days				
Pregnancy index, first 2 litters (control 76–100)	100 / 76	94 / 88	100 / 88	94 / 81	94 / 82	71 / 59	0 / 0				
Cumulative days to litter	↔	↔	↔	↔	↔	↔	no litters				
F₁ Females											
Estrous cycle effects	↔	↔	↔	↔	↔	↔	↑0.4 days				
Body weight at necropsy	↔	↔	↔	↔	↔	↔	↓19%				
Relative uterine weight	↔	↔	↔	↔	↔	↔	↑36%				
Relative ovarian weight	↔	↔	↔	↔	↔	↔	↑35%				
F₁ Breeder males											
Body weight at necropsy	↔	↔	↔	↔	↔	↔	↓16%				
Absolute organ weight											
Cauda epididymis	↔	↔	↔	↔	↔	↓37%	↓62%	6532	3576	5970	2224
Epididymis	↔	↔	↔	↔	↔	↓35%	↓55%	6764	3896	6184	2226
Testis	↔	↔	↔	↔	↔	↓51%	↓80%	6577	1693	5940	511
Seminal vesicles	↔	↔	↔	↔	↔	↔	↓29%				
Ventral prostate	↔	↔	↔	↔	↔	↓28%	↔	7056	3062	6998	2725
Dorsolateral prostate	↔	↔	↔	↔	↔	↔	↓29%				
No. with small testes	0	0	0	0	0	8/10	10/10				
No. with small epididymides	0	0	0	0	0	2/10	0				
F₁ Non-breeder males											
Body weight at necropsy	↔	↔	↔	↔	↔	↓9%	↓21%	6752	6408	1634	1229
Absolute organ weight						↓20%					
Epididymis	↔	↔	↔	↔	↔		↓54%	6845	3349	5787	797
Seminal vesicles	↔	↔	↑9%	↔	↔	↔	↔				
Cauda epididymis	↔	↔	↔	↔	↔	↓20%	↓44%	6802	4188	5669	1450
Testis	↔	↔	↔	↔	↔	↓34%	↓80%	2945	2584	1221	946
Relative organ weight											
Epididymis	↔	↔	↔	↔	↔	↔	↓42%	6474	5814	3088	1965

Parameter	Dose in feed (ppm)							Benchmark dose (ppm)[a]			
	10	30	100	300	1000	7500	10,000	BMD_{10}	$BMDL_{10}$	$BMD_{1\,SD}$	$BMDL_{1\,SD}$
Testis	↔	↔	↔	↔	↔	↓28%	↓75%	3895	3253	1903	1312
Cauda epididymis	↔	↔	↔	↔	↔	↔	↓33.8%				
No. with small:											
testes	0	0	0	3/45	0	9/30	21/21				
epididymides	0	0	0	2/45	0	0	21/21				
seminal vesicles	0	0	0	2/45	0	0	1/21				
ventral prostates	0	0	0	0	3/43	1/30	1/21				
caudae epididymides	0	0	0	1/45	0	0	21/21				
Spermatid/testis	↔	↔	-	↔	-	↓69%	↓100%	5834	1329	5534	916
Sperm/cauda	↔	↔	↔	↔	↔	↓61%	↓99.8%	6771	2865	6663	2472
Epididymal sperm density	↔	↔	↔	↔	↔	↔	↓99.6%	6389	2819	6181	2310
F_1 Matings											
Live F_2 pups/litter	↔	↔	↔	↔	↔	↔					
Live pup weight	↔	↔	↔	↔	↔	↓10%		7496	7119	5996	1762
adjusted for litter size	↔	↔	↔	↔	↔	↓11%		7245	6708	2123	1247
Male F_{2a} anogenital distance	↔	↔	↔	↔	↔	↓13%		5749	4582	1093	963
Male F_{2c} anogenital distance	↔	↔	↔	↔	↔	↓18%		5375	3284	3702	2517
Male pup weight PND 1	↔	↔	↔	↔	↔	↓13%		7192	4135	6788	2649
Male pup weight PND 4	↔	↔	↔	↔	↔	↓22%		6960	2715	6362	2365
Female pup weight PND 1	↔	↔	↔	↔	↔	↓17%		5801	3505	6669	2036
Female pup weight PND 21	↔	↔	↔	↔	↔	↓28%		5097	2144	4638	2209
Male pup survival PND 1–21	↔	↔	↔	↔	↔	↓20%		7004	2992	7082	3504
Combined survival PND 1–21	↔	↔	↔	↔	↔	↓19%		7293	3057	6924	3130
F_2 Males and females											
Age at vaginal opening	↔	↔	↔	↔	↔	↑6 days					
preputial separation (days)	↑1.9	↑1.0	↑1.4	↑0.7	↑6.5						
testes descent (days)	↔	↑1.4	↑0.8	↑0.4	↑3.4						
Pregnancy index, 1st 2 litters (control 100)	100	100	100	100	100	53(↓)					
	100	100	100	100	94	47(↓)					
Cumulative days to deliver	↔	↔	↔	↔	↔	↔					

Parameter	Dose in feed (ppm)							Benchmark dose (ppm)[a]			
	10	30	100	300	1000	7500	10,000	BMD_{10}	$BMDL_{10}$	$BMD_{1\,SD}$	$BMDL_{1\,SD}$
No. cycling rats	↔	↔	↔	↓29%	↔	↔					
No. litters/pair	↔	↔	↔	↔	↔	↓					
F_2 Males (breeders)											
Body weights at necropsy	↔	↔	↔	↔	↔	↓14%		7346	4638	7003	3233
Absolute organ weight											
Epididymis	↔	↔	↔	↔	↔	↓36%		6593	1846	4490	678
Seminal vesicles	↔	↔	↔	↔	↔	↓24%		2916	2009	2209	1705
Testis	↔	↓10%	↔	↔	↔	↓60%		5874	1329	2408	1300
Cauda epididymis	↔	↔	↔	↔	↔	↓63%		6493	1641	6711	2432
Relative testis weight	↔	↔	↔	↔	↔	↓53%		2334	1224	1815	1065
No. with small:											
Testes	0	0	0	0	0	8/10					
Epididymides	0	0	0	0	0	8/10					
Caudae epididymides	0	0	0	0	0	8/10					
F_2 Males (non-breeders)											
Body weight at necropsy	↔	↔	↔	↔	↔	↓14.5%		7190	4446	6289	3153
Absolute organ weight											
Epididymis	↔	↔	↔	↔	↔	↓27%		4350	2269	2574	1270
Testis	↔	↔	↔	↔	↔	↓49%		1788	1362	987	758
Cauda epididymis	↔	↔	↔	↔	↔	↓32%		2532	2005	1132	895
Relative organ weight											
Testis	↔	↔	↔	↔	↔	↓40%		1948	1470	1132	1003
Cauda epididymis	↔	↔	↔	↔	↔	↓20%		4187	2714	1718	1472
No. with small:											
Testes	0	0	0	1/21	1/25	11/20					
Epididymides	0	0	0	1/21	1/25	7/20					
Caudae epididymides	0	0	0	1/21	1/25	6/21					
Spermatid/testis	↔	↔	↔	↔	↔	↓74%		2885	1092	2043	1161
Sperm/cauda	↔	↔	↔	↔	↔	↓72%		1795	935	1374	854
Epididymal sperm density	↔	↔	↔	↔	↔	↓64%		1158	897	1830	1542
Percent motile sperm	↔	↔	↔	↔	↔	↓25%		3825	2038	554	412

Parameter	Dose in feed (ppm)							Benchmark dose (ppm)[a]			
	10	30	100	300	1000	7500	10,000	BMD_{10}	$BMDL_{10}$	$BMD_{1\,SD}$	$BMDL_{1\,SD}$
F_2 Matings											
Live F_3 pups/litter	↔	↔	↔	↔	↔	↔					
Live pup weight	↔	↔	↔	↔	↔	↔					
Male anogenital distance, F_{3a}	↔	↔	↔	↔	↔	↓13%		7322	4638	6846	2661
Pup weight (PND 1–21)	↔	↔	↔	↔	↔	↔					
Pup survival (PND 1–21)	↔	↔	↔	↔	↔	↔					
F_3 Females											
Age at vaginal opening	↔	↔	↔	↔	↔	↑6					
F_3 Males											
Retained nipples	↔	↔	↔	↔	↔	↑11%					
Age at preputial separation	↔	↔	↔	↔	↔	↑5.1					
Age of testes descent	↔	↔	↔	↔	↔	↑2.5					
Body weights at necropsy	↔	↔	↔	↔	↔	↔					
Absolute organ weights											
Dorsolateral prostate	↔	↔	↔	↔	↔	↓41%		1926	1401	2204	1840
Testis	↔	↔	↔	↔	↔	↓45%		1960	1399	1060	851
Relative organ weights											
Testis	↔	↔	↔	↔	↔	↓48%		1870	1307	1330	1097
Epididymis	↔	↔	↔	↔	↔	↓35%		3223	1625	2332	1285
Spermatid/testis	↔	↔	↔	↔	↔	↓79%		1159	782	1100	695
Sperm/cauda	↔	↔	↔	↔	↔	↓95%		787	728	1188	970
Epididymal sperm density	↔	↔	↔	↔	↔	↓94%		797	750	1108	933

[a]Calculated by CERHR. See Table 20 for definitions and a discussion of the use of benchmark dose in this report. The 10,000 ppm dose group was not included in the benchmark dose calculations because it used a different control than the other dose groups. Benchmark dose was calculated only when there was a significant treatment effect according to the study authors' analysis.
From (145).

Table 30 . Reproductive Crossover Breeding Study

Parameter		F_1 DEHP treatment groups		F_2 DEHP treatment groups	
	Dam	10,000 ppm ×	Naïve ×	7500 ppm ×	Naïve ×
	Sire	naïve	10,000 ppm	naïve	7500 ppm
Pregnancy index		↑	↓ (0 pregnant)	↔	↓
Fertility index		↑	↓ (0 fertile)	↔	↓
Mating index		↔	↓ (0 mated)	↔	↔
Implantation sites		not reported	↓97.9%	not reported	↓54.5%
Adjusted live male pup weight		↓13.6%	not applicable	↓8.2–12.3%	↔
Male pup anogenital distance		↓16.9%	not applicable	↓11.5%	↔
Female anogenital distance/bw		↑17.6%	not applicable	↔	↔
Live female pups/litter		↔	not applicable	↔	↓31.0%

From *(145)*.

4.2.4 Abstracts

CERHR retrieved several abstracts reporting reproductive toxicity associated with DEHP exposure. Although information from abstracts is not considered by the Expert Panel in reaching final conclusions, the abstracts are briefly summarized for the sake of completeness.

Boekelheide et al. *(146)* presented an abstract on the use of mutant mice to investigate the MEHP mechanism of testicular toxicity. Knockout mice deficient in fasL, p53, or both were protected against testicular toxicity after treatment with MEHP. The authors concluded that the apoptotic response of mouse testis to MEHP is at least partially dependent on fasL and p53. **[The Expert Panel notes that the results for fasL-deficient mice reported in this abstract appear similar to the results in Richburg et al. *(130)*, discussed above, from the same laboratory.]**

Noriega et al. *(147)* treated male Sprague-Dawley rats on PND 23–43 or 44 with DEHP 0, 100, 300, or 900 mg/kg bw/day **[route not specified]**. There were reductions in the 300 and 900 mg/kg bw/day groups in testis, seminal vesicle, Cowper gland, levator ani, and bulbocavernosus muscle weights compared to the controls. Testicular testosterone (basal and stimulated) was reduced in these 2 groups, and serum testosterone was reduced in the 900 mg/kg bw/day group. There was a reduction in the proportion of males with complete preputial separation in the 300 and 900 mg/kg bw/day groups compared to the controls. Female rats dosed from PND 23 concurrently with males showed no difference in time to vaginal opening in the 900 mg/kg bw/day group compared to the corn-oil control group.

Nakajima et al. *(148)* gave wild-type and PPARα-null mice DEHP in the diet for 4 weeks at exposure levels of 0 or 0.05%, following which the animals were mated within dose and genetic-strain groups. Two generations (F_1 and F_2), produced without direct exposure to DEHP, showed decreases in number of pups born and in pups surviving to 16 weeks of age. Only wild-type mice were affected. In another experiment, dietary DEHP exposures at 0, 0.01, and 0.05% were used, producing an increase in resorptions and a decrease in newborn survival among wild-type mice born to treated animals. There were no alterations in parental reproductive organs.

Kang and Lee *(149)* treated male Sprague-Dawley rats orally for 4 weeks with phthalate diesters at 500 mg/kg bw/day or phthalate monoesters at 250 mg/kg bw/day. Animals were evaluated for body weight, testicular and epididymal weights, epididymal sperm count, and sperm motion parameters. There were no significant effects of treatment on body or reproductive organ weight. The order (strongest to weakest) for effects on sperm motility for the diesters was DEHP > DnOP > DEP > DUP > DIDP > BBP, and for the monoesters was MBuP > MEP > MEHP. **[Abbreviations were not defined but are assumed to be: DnOP = di-n-octyl phthalate; DEP**

= diethyl phthalate; DUP = diundecyl phthalate; DIDP = diisodecyl phthalate; BBP = butyl benzyl phthalate; MBuP = monobutyl phthalate; MEP = monoethyl phthalate.]

Jayes and Davis *(150)* cultured granulosa cells from PMSG-stimulated immature rats. Medium containing FSH and testosterone was treated with MEHP at 0, 0.001, 0.01, 0.1, 1, 2, or 5 μM. Estradiol was assayed in media at 51 hours, and aromatase was measured in cells exposed to 0, 1, or 5 μM MEHP. Estradiol and aromatase were decreased by MEHP at 1 or 5 μM compared to controls.

4.3 Utility of Reproductive Toxicity Data

Since the initial CERHR Expert Panel report on DEHP, a human time to pregnancy study, which bears directly on fertility, was published. Another human study evaluates a putative association between DEHP exposure and endometriosis, a disease that may have fertility implications; however, this study does not permit an evaluation of possible DEHP effects on fertility. There are several in vitro studies on the effects of MEHP on rat granulosa cells, bovine oocytes, mouse Leydig tumor cells, mouse Sertoli cells, and rat, mouse, or guinea pig seminiferous tubule sections. These studies may contribute to an understanding of possible mechanisms of DEHP reproductive toxicity but do not directly evaluate reproductive function. A single in vivo female reproductive toxicity study evaluated the effects of sc DEHP on ovulation induction in intact rats. Male reproductive toxicity studies in rodents have been designed to explore the time course and mechanisms of DEHP or MEHP testicular toxicity. Apoptosis initiation in germ cells, zinc depletion, and oxidative stress have been investigated, as well as specific alterations in enzyme activities involved in testicular steroidogenesis. Fertility has been directly evaluated in male and female rats in a multigeneration study and in a Reproductive Assessment by Continuous Breeding study.

4.4 Summary of Reproductive Toxicity Data

4.4.1 Human Data

Modigh et al. *(114)* evaluated time to pregnancy in the partners of 182 unexposed pregnancies fathered by workers who were not exposed during the time leading up to pregnancy or by office staff and unexposed male partners of female workers, 100 low-exposure pregnancies fathered by men with estimated non-zero DEHP exposures < 0.1 mg/m^3, and 44 high-exposure pregnancies fathered by men with DEHP exposures ≥ 0.1 mg/m^3. Median time to pregnancy was 3.0 months in the unexposed group, 2.25 months in the low-exposure group, and 2.0 months in the high-exposure group. The crude and adjusted fecundability ratios for the exposed pregnancies were all close to 1.0, and the 95% confidence intervals all overlapped unity. There was no significant effect of restricting the analysis to the first pregnancy of couples with more than 1 pregnancy or of excluding pregnancies conceived by employed women. Excluding couples with known fertility problems did not influence the findings. The authors concluded that there was no evidence of a DEHP-associated prolongation in time to pregnancy, although they recognized that there were few highly exposed men in their sample; the mean DEHP exposure level for men in the study was less than 0.5 mg/m^3.

4.4.2 Experimental animal data

4.4.2.1 Female

Sekiguchi et al. *(123)* evaluated the number of ova ovulated in response to 15 or 30 IU equine chorionic gonadotropin as an assay of female reproductive toxicity in F344 rats. Based on small sample size and inadequate statistical methods, this study was considered inadequate for the evaluation process.

4.4.2.2 Male
Male reproductive toxicity data in experimental animals are summarized in Table 31.

The time course of testicular injury after a single gavage dose of MEHP was evaluated by
Dalgaard et al. *(133)*. Rats at 28 days of age were given MEHP in corn oil at 0 or 400 mg/kg bw.
Rats given corn oil were killed 2 hours later, and rats given MEHP were killed 3, 6, or 12 hours
later. Light microscopy showed germ cell sloughing beginning 6 hours after MEHP treatment.
Collapse of vimentin filaments in the Sertoli cell cytoplasm and perinuclear condensation were
evident by immunostaining at 3 hours and persisted throughout the study. There were no
detectable treatment effects on number of apoptotic cells per tubule or DNA laddering patterns
over the 12-hour duration of the study.

Timing of DEHP effects was also evaluated by Park et al. *(134)* to investigate the role of altered
zinc homeostasis in testicular toxicity. Rats were administered DEHP in corn oil at 0 or 2
g/animal daily for up to 14 days. Testes were evaluated after 1, 2, 3, 5, 7, 10, or 14 days of
treatment. Effects on testis weight were noted after 7 days (the first time point after day 2 for
which a control comparison was available). Histologic evidence of apoptosis was evident before
day 3, and necrosis was severe by day 7. Testicular zinc decreased beginning on treatment day 3,
but zinc-transporter-1 mRNA was not altered until day 14. The authors concluded that reduced
testicular zinc was probably a result of testicular toxicity after DEHP treatment rather than a
mediator of DEHP toxicity.

Kasahara et al. *(135)* evaluated the role of oxidative stress in DEHP-associated testicular toxicity
in rats. DEHP was given by gavage in corn oil at 0, 1000, or 2000 mg/kg bw/day for 7 days. Total
glutathione, low-molecular-weight thiols, and ascorbic acid were decreased in testis after
treatment with DEHP 1000 mg/kg bw/day. Glutathione and low-molecular-weight thiols were
increased and ascorbic acid was unchanged in liver and kidney at both DEHP dose levels.
Glutathione and catalase were increased in testis at both doses of DEHP and unchanged or
decreased in liver and kidney. The release of reactive oxygen species was reported to increase in
a time-dependent manner in cells derived from animals treated with DEHP 2000 mg/kg bw/day.
This increase was prevented by addition of superoxide dismutase, catalase, or sodium azide,
suggesting to the authors that the superoxide radical and hydrogen peroxide were increased by
DEHP treatment. TUNEL-positive cells were reported to be markedly increased in the testes of
animals that had been treated with DEHP 2000 mg/kg.

Three studies evaluated the importance of fas in the germ cell apoptosis associated with DEHP
and MEHP testicular toxicity in rodents. Ichimura et al. *(131)* treated DDY mice with DEHP at
up to 4000 mg/kg bw (single oral dose) and evaluated testes 12 hours later. Immunochemical
methods were used to localize fas and caspase-3 to the middle and outer portion of the epithelium
and fasL to a distribution consistent with Sertoli cell processes and spermatocytes. TUNEL
staining of nuclei was localized to the middle layer of the seminiferous epithelium. Two other
studies *(130, 132)* compared the response of wild-type and mutant *gld* mice to MEHP 1000
mg/kg as a single dose. The *gld* mutant have a nonfunctional form of fasL. TUNEL labeling and
fas expression were increased as soon as 3 horus after treatment in wild-type mice but were
reduced in mutant mice. In 1 study (Giammona, 2002 #160}, expression of the death receptor
DR5 in testis increased as early as 1.5 hours after MEHP administration.

Testicular steroidogenesis in rats treated with DEHP was evaluated in 3 studies *(80, 138, 140)*.
Kim et al. *(138)* found a decrease in testicular aromatase and in the hydroxylation of testosterone
after DEHP 100 mg/kg bw/day for 5 days. There was no alteration in serum testosterone at this
dose, leading the authors to conclude that the enzyme activities were more sensitive measures of
DEHP toxicity than serum testosterone. Because the dosing schedule included a recovery period,

the results could not be interpreted by the Expert Panel for use in the evaluation. Akingbemi et al. *(80)* evaluated the age-related sensitivity of rat testicular steroidogenesis to DEHP disruption. Prepubertal male Long-Evans rats were exposed by gavage to DEHP on PND 21–34, 35–48, or 21–48, and young adult rats were treated on PND 62–89. DEHP was administered in corn oil at 0, 1, 10, 100, or 200 mg/kg bw/day. Treatment of prepubertal rats on PND 21–34 or PND 35–48 did not produce alterations of serum LH or testosterone, but treatment on PND 21–48 produced a dose-related increase in serum LH and testosterone and in interstitial fluid testosterone that was statistically significant at a DEHP dose level of 10 mg/kg bw/day. Leydig cells isolated from rats after DEHP treatment on PND 21–34 showed a decrease in basal and LH-stimulated testosterone production ex vivo (LOAEL 100 mg/kg bw/day). Leydig cells isolated from rats after DEHP treatment on PND 35–48 showed decreased basal and LH-stimulated testosterone production ex vivo (LOAEL 10 mg/kg bw/day) and decreased activities of enzymes in the testosterone synthesis pathway. The most sensitive enzyme was 17β-hydroxysteroid dehydrogenase. Leydig cells isolated from prepubertal rats that had been treated on PND 21–48 showed an increase in basal and LH-stimulated testosterone production with a LOAEL of 10 mg/kg bw/day. In adult rats, there were no effects of DEHP treatment at any tested dose. The authors identified 1 mg/kg bw/day as a NOAEL and 10 mg/kg bw/day as a LOAEL.

In a follow-up study *(140)*, rats were gavaged on PND 21–90 or PND 21–120 with DEHP in corn oil at 0, 10, or 100 mg/kg bw/day. Leydig cells were isolated from testes and cultured for 3 hours with and without maximally stimulating concentrations of ovine LH. Serum LH and testosterone were increased compared to controls, and basal and stimulated testosterone production in Leydig cell culture was significantly reduced. On PND 90, these effects were seen at both DEHP doses, but on PND 120, only the 100 mg/kg bw/day group was affected. The study authors believed the results consistent with Leydig cell hyperplasia because serum testosterone was increased in the presence of a reduction in testosterone production per Leydig cell. Leydig cell hyperplasia was identified in both dose groups using markers of cell division (PCNA, cyclins D_3 and G_1), incorporation of tritiated thymidine in cultured Leydig cells, and counting of Leydig cells in microscopic sections of testis. In another experiment, male rats were gavaged with DEHP in corn oil at 0, 10, or 100 mg/kg bw/day from PND 21 to PND 48 or 90. Serum estradiol was increased on PND 48 in rats in both DEHP exposure groups. Basal estradiol production in cultured Leydig cells was increased in the 100 mg/kg bw group, and LH-stimulated estradiol production was increased in both DEHP dose groups on PND 48. Aromatase mRNA was also increased in Leydig cells from both DEHP-treated groups. The authors concluded that DEHP increases Leydig cell populations associated with chronically increased LH and testosterone levels, and that a decrease in testosterone and increase in estradiol synthesis (per cell) was consistent with induction of aromatase activity in Leydig cells.

4.4.2.3 Male and Female
Reproductive toxicity data in male and female rats are summarized in Table 32.

Schilling et al. *(113)* performed a 2-generation reproductive study of DEHP in Wistar rats. DEHP (99.7% pure) was administered in feed at 0, 1000, 3000 and 9000 ppm, resulting in estimated DEHP intakes of 0, 113, 340, and 1088 mg/kg bw/day. Females littered and raised their own pups. On PND 4, litters were standardized to 8 (4 males and 4 females where possible). Treated feed was provided throughout the gestation and lactation period, and pups were weaned to the diet assigned to their parents. On PND 28, 10 F_2 offspring/sex/dose group were evaluated by functional observation battery and motor activity testing and an additional 10 F_2 offspring/sex/dose group were evaluated in a water maze test of learning and memory. Estrous cycle was monitored daily for all F_0 and F_1 females for at least 3 weeks prior to mating. Cauda epididymal sperm were assessed for motility, morphology, and head count in F_0 and F_1 males at necropsy. Testicular sperm head count was also performed. Male pups were evaluated on PND 12

and 13 for the presence of nipples and areolae. Day and weight at vaginal opening and preputial separation were assessed in pups. The high-dose level (9000 ppm) was associated with a decrease in feed consumption and weight gain at several intervals during the study. F_2 pups in the high-dose group were smaller and gained less weight from birth through the assessment of functional observation battery and water maze testing. Grip strength was reduced in males and hind-limb splay was reduced in both sexes in the high-dose group. There were no other treatment-related findings in the functional observation battery or in the water maze. Differential ovarian follicle counts of F_0 and F_1 adults showed a deficit in growing follicles and corpora lutea in the high-dose group **[expressed per follicle]**. The authors concluded that reproductive performance and fertility were affected at the 9000 ppm dose level (1088 mg/kg bw/day) with a NOAEL of 3000 ppm (340 mg/kg bw/day), and that developmental toxicity was noted at 3000 and 9000 ppm, with an increase in stillbirth, an increase in PND 0–4 pup mortality, retardation of F_2 pup body weight, altered male anogenital distance, and retained nipples/areolae. A delay in sexual maturation was also noted in F_1 offspring at 9000 ppm. The NOAEL for developmental toxicity was considered by the study authors to be 1000 ppm as was the NOAEL for systemic toxicity.

The NTP *(145)* sponsored a multigeneration continuous-breeding study in rats. After a preliminary dose-ranging study, 17 Sprague-Dawley rats/sex/group were fed diets containing 1.5 (control group exposed to background DEHP levels), 10, 30, 100, 300, 1000, or 7500 ppm DEHP (99.8% pure) from the first day of the study until the day of necropsy. Due to a described lack of reproductive effects in the first litter produced, the study was repeated with 2 additional doses, 1.5 (control) and 10,000 ppm. Ranges of DEHP intake in the F_0, F_1, and F_2 animals were estimated at 0.09–0.12, 0.47–0.78, 1.4–2.4, 4.8–7.9, 14–23, 46–77, 392–592, and 543–775 mg/kg bw/day. At about 5 weeks of age, F_0 rats were fed the DEHP-containing diets for 6 weeks prior to mating and were then cohabitated for 9 weeks. The first 2 litters delivered during the cohabitation period (F_{1a} and F_{1b}) were counted, weighed, assessed for anogenital distance, and then discarded. The third litter (F_{1c}) was raised by the dam. Following weaning of pups, vaginal cytology was monitored in F_0 females for 14 days. After completion of crossover studies described below, at least 10 F_0 rats sex/group were necropsied. Sperm analyses were conducted, and organs were collected for histopathological evaluation. F_1 pups were counted, weighed, and examined for anogenital distance and nipple retention during the lactation period. On PND 16, 1 female per litter was evaluated for vaginal opening, and a second was selected for F_1 mating. One male per litter was selected for mating, and 4 or 5 males per litter were evaluated for testicular descent and preputial separation. At weaning on PND 21, pups were given diets containing the same DEHP concentrations as their parents. On PND 81, the F_1 rats chosen for mating (17/sex/group) were randomly assigned to breeding pairs (preferably non-sibling) and cohabited for 9 weeks. The study conducted in F_0 parents and F_1 offspring was repeated in F_1 parents and F_2 offspring, except that no F_3 offspring were mated. Selected F_{3c} males were necropsied on PND 63–64 and selected females on PND 60–74.

During numerous time periods of the study and especially at necropsy, body weight gains were decreased in rats from the 7500 and 10,000 ppm groups. Dam body weights during delivery and lactation were decreased by 8–20% in the F_0 10,000 ppm group. Increases and decreases in feed intake were observed at most dose levels. In the F_0 7500 and 10,000 ppm groups, feed intake was decreased during lactation. The liver was identified as a target of toxicity with increases in liver weight and hepatocellular hypertrophy observed at dose levels ≥1000 ppm. Changes in organ weights and lesions were also observed in the kidney at ≥7500 ppm and the adrenal gland at 10,000 ppm.

Reproductive toxicity findings in all generations of rats are summarized in Table 29. The only reproductive effects observed in the F_0 parents occurred at 10,000 ppm and included decreases in sperm counts and velocity, reductions in testis and epididymis weights, and increased numbers of

rats with small testes. Histopathological findings associated with small testes included minimal-to-marked atrophy of seminiferous tubules characterized by loss of germ cells. The lowest dose level producing dose-related effects in F_1 offspring was 7500 ppm, and those effects included decreases in number of live pups/litter, reduced male anogenital distance, and delays in vaginal opening, preputial separation, and age at testicular descent. Additional effects noted in the F_1 offspring from the 10,000 ppm group included decreased live pup weight at birth and during the lactation period and increased ratio of female anogenital distance to body weight.

Fertility was compromised in the F_1 rats from the 10,000 ppm group, which did not produce any viable litters. Other effects observed in F_1 parents were similar to those observed in F_0 parents but occurred at lower dose levels. Histopathological findings observed in all animals of the 7500 and 10,000 ppm groups were consistent with those observed in the F_0 generation and included minimal-to-marked seminiferous tubule atrophy and occasional sperm release failure. Additional reproductive effects observed in F_1 rats were reduced sperm counts at 7500 ppm and higher and increased uterus and ovary weights at 10,000 ppm. In the F_2 pups, delays in preputial separation and testicular descent occurred at each dose level. Other effects occurring in F_2 pups of the 7500 ppm group included delayed vaginal opening and reductions in live pup weight at birth and during the lactation period, male anogenital distance, and survival during the lactation period.

In the F_3 offspring, seminiferous tubule atrophy was observed in 10/10 males from the 7500 ppm group. All other reproductive effects in F_2 adults occurred at 7500 ppm and included decreases in pregnancy index, the number of litters per pair, male reproductive organ weights, sperm counts, and sperm motility. The F_3 generation was the only generation in which there was an increase in males with nipples. Other F_3 effects included reduced sperm counts and weights of dorsolateral prostate, testis, and epididymis at 7500 ppm.

The study authors discussed the relevancy of small male reproductive organ sizes observed in both F_1 and F_2 rats of the 300 ppm groups. They noted that although incidences were low, the effects were consistent with phthalate-induced toxicity. The incidence of small testes and epididymides exceeded historical control data from the laboratory. Therefore, the study authors considered the effects as potentially treatment-related. However, the study authors concluded that the overall significance of the effects cannot be determined due to lack of histopathological data and lack of adverse reproductive effects at 300 and 1000 ppm. Using crossover studies, the male was identified as the sex in which adverse fertility effects occurred.

The study authors concluded, "The findings obtained in this study indicate that DEHP is clearly a reproductive and developmental toxicant at 7500 and 10,000 ppm based upon changes in fertility and pregnancy indices, litter data, sperm parameters, sexual development, and/or histopathological changes in testes." Intake at 7500 ppm was estimated at 392–592 mg/kg bw/day, and intake at 10,000 ppm was estimated at 543–775 mg/kg bw/day. Based on the incidence of testicular abnormalities, the Expert Panel considered 300 ppm to be an effect level, giving a NOAEL of 100 ppm, about 3–5 mg/kg bw/day. **[The lowest BMD_{10} was 787 ppm based on F_3 sperm/cauda. Extrapolating from the authors' estimates of intakes at 1000 mg/kg bw/day, this intake level is 36–61 mg/kg bw/day. The lowest $BMD_{1\,SD}$ is 554 ppm based on F_2 percent motile sperm. Extrapolating from the authors' estimates of intakes at 300 ppm, this intake level is 9–15 mg/kg bw/day. The Expert Panel notes that Sertoli cell vacuolation, which was the endpoint driving the LOAEL in the original CERHR Expert Panel evaluation of DEHP, was not increased by DEHP treatment in this study.]**

Table 31. Summary of Male Reproductive Toxicity Data from Studies in Rats and Mice

Species and dosing	Most sensitive outcome	Effect levels (mg/kg bw/day)	Reference
Single dose-level studies (excluding control)			
Mice, C57Bl6 wild-type and *gld* mutant. MEHP 1000 mg/kg bw × 1 by gavage	TUNEL labeling greater in wild-type than fasL-deficient mutant	1000	Richburg et al. *(130)*
Mice, C57Bl6 wild-type and *gld* mutant. MEHP 1000 mg/kg bw × 1 by gavage	Apoptotic response decreased in fasL-deficient mutant compared to wild-type mice	1000	Giammona et al. *(132)*
Rat, Sprague-Dawley. MEHP 1000 mg/kg bw × 1 by gavage	Increased expression of death receptors	1000	Giammona et al. *(132)*
Rat, Wistar. MEHP 400 mg/kg bw × 1 by gavage	Germ cell sloughing, collapse of Sertoli cell vimentin filaments 3 hours after treatment	400	Dalgaard et al. *(133)*
Rat, Sprague-Dawley. DEHP 2 mg/rat/day **[19,000–29,000 mg/kg bw/day]** × 1–14 days	Decreased body and testis weight, testis histologic evidence of apoptosis beginning treatment day 3	**[19,000–29,000]**	Park et al. *(134)*
Multiple dose levels			
Rat, Wistar. DEHP 0, 1000, or 2000 mg/kg bw/day by gavage × 7 days	Decreased total glutathione, low molecular weight thiols, and ascorbic acid in testis	LOAEL 1000	Kasahara et al. *(135)*
Rat, Wistar. DEHP 0, 100, or 1000 mg/kg bw/day by gavage × 5 days	Decreased aromatase and increased CYP2C11 and CYP3A2 testosterone hydroxylation in testis	LOAEL 100[a]	Kim et al. *(138)*
Rat, Long-Evans. DEHP 0, 1, 10, 100, or 200 mg/kg bw/day by gavage × 14 or 28 days	Decreased 17α-hydroxylase in testis, altered ex vivo Leydig cell testosterone synthesis	LOAEL 10 NOAEL 1	Akingbemi et al. *(80)*
Rat, Long-Evans. DEHP 0, 10, or 100 mg/kg bw/day by gavage × 70 or 100 days	Increased serum LH and testosterone, decreased ex vivo Leydig cell testosterone synthesis (PND 90)	LOAEL 10	Akingbemi et al. *(140)*

[a]The use of a recovery period in the dosing schedule prevented the Expert Panel from being able to interpret the results.

Table 32. Summary of Reproductive Toxicity Studies Involving Male and Female Rats

Species and dosing	Most sensitive outcome	Effect levels, ppm	Reference
Rat, Wistar. DEHP in feed at 0, 1000, 3000, or 9000 ppm, giving DEHP intakes estimated as 0, 113, 340, or 1088 mg/kg bw/day × 2 generations	Increased abnormal sperm, decreased male fertility, decreased testis size, decreased litter size and viability, deficit in growing follicles and corpora lutea	LOAEL 9000 **[~1088 mg/kg bw/day]** NOAEL 3000 **[~340 mg/kg bw/day]** **[Lowest BMD$_{10}$[a] 2325 ppm (~263 mg/kg bw/day) based on F$_2$ pup survival PND 0–4]**	Schilling et al. *(113)*
Rat, Sprague-Dawley. DEHP in feed at 1.5, 10, 30, 100, 300, 1000, 7500, or 10,000 ppm (the 10,000 ppm dose was run separately with its own 1.5-ppm control). Rats were treated over 3 generations using a continuous-breeding protocol for up to 3 litters/pair. DEHP intake was estimated at 0.09–0.12 and 0.47–0.78 for the 2 controls, 0.09–0.12, 0.47–0.78, 1.4–2.4, 4.8–7.9, 14–23, 46–77, 392–592, and 543–775 mg/kg bw/day.	Fertility and pregnancy indices, litter data, sperm parameters, sexual development, and histopathological changes in testes.	Authors' LOAEL 7500 **[392–592 mg/kg bw/day]**, although occasional effects at 300 ppm were considered possibly treatment-related. The Expert Panel considers 300 ppm to be an effect level and the NOAEL to be 100 ppm (3–5 mg/kg bw/day) **[Lowest BMD$_{10}$[a] 787 ppm (36–61 mg/kg bw/day) based on F$_3$ sperm/cauda; lowest BMD$_{1\ SD}$ 554 ppm (9–15 mg/kg bw/day) based on F$_2$ percent motile sperm.**	National Toxicology Program *(145)*

[a]Calculated by CERHR. See Table 20 for definitions and a discussion of the use of benchmark dose in this report.

Conclusions from the original Expert Panel evaluation

The original Expert Panel report on DEHP contained conclusions in Section 5. These conclusions have been extracted and reproduced below, with the section numbering as found in the original document. The references listed in the conclusion are listed, and the table to which the conclusions refer is reproduced, numbered 76 as in the original.

5.1.4. Reproductive toxicity
There are no data on the reproductive toxicity of DEHP or its major metabolites in humans.

5.1.4.1.1. Females. There are data that indicate that oral exposure to DEHP can affect reproductive processes in rats and mice. Data presented in [168] clearly show adverse functional effects at a dietary dose of 425 mg/kg bw/day where complete infertility was observed, although the design did not allow conclusions as to whether males, females, or both sexes contributed to reduced number of pups and pup viability. Only the data from [219] come close to evaluating broadly the effects on the female tract, and this study examined only a high dose. Davis et al. [219] showed clear effects on estradiol synthesis and ovulation in rats at 2000 mg/kg bw/day. No histopathological structural changes were seen in the uterus or vagina. Further, there are no studies that have evaluated adult female reproductive structure and function after prenatal exposure. Current data are not adequate to confidently ascribe a NOAEL or LOAEL for female reproduction (Table 76).

5.1.4.1.2. Males. The oral exposure studies of Lamb [168] and Schilling et al. [169] are sufficient to conclude that DEHP is a reproductive toxicant in male rats and mice. In the Lamb study [168], only the control and high-dose F0 mice were necropsied; thus, it is not known if the reduced fertility at ~141 mg/kg bw/day (the middle dose) is partly male-mediated resulting from testicular damage. Schilling et al. [169] reported effects (androgen mediated and testicular lesions) at 1060 mg/kg bw/day in rats, and no effects (i.e. a NOAEL) at ~339 mg/kg bw/day. The study of Lamb [168] has a NOAEL of ~14 mg/kg bw/day (Table 76)… it is clear from the existing data that testicular pathology and reduced sperm numbers are consistent effects. The data are sufficient to conclude that DEHP is a reproductive toxicant in male rats, mice, ferrets, and guinea pigs when administered orally. There is greater uncertainty when determining lowest-adverse and no-adverse effect levels of exposure. The two studies [75,169] that used peripubertal dosing (believed to be the most sensitive period for causing adverse effects) and evaluated the rats when at, or close to, maturity (believed to be the most sensitive period for observing adverse effects) present markedly different NOAELs. The expert panel could not confidently reconcile these differences (~339 versus 3.7 mg/kg bw/day). Confidence in results observed at a given dose in [169] is eroded slightly by the small group size. The study of Poon et al. [75] was thorough in its design and execution, including verification of dose. While there were 20 animals per dose group, only 10 were males; the study design did not incorporate measures of reproductive function. The authors were clear in asserting that ". . . the mild Sertoli cell vacuolation at 500 ppm (~38 mg/kg bw/day) should be considered an adverse effect." The expert panel finds there is a reasonable basis for such a conclusion (i.e. a LOAEL). The NOAEL from this study is therefore 3.7 mg/kg bw/day. When comparing the NOAELs from [75,168], 3.7 mg/kg bw/day versus 14 mg/kg bw/day, it is reasonable to conclude that these values are indistinguishable given the wide dose spacing and inherent biological variability in the endpoints. It is the panel's view that the existing data support a NOAEL within the range of 3.7−14 mg/kg bw/day for oral exposure in rats.

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Table 76 from the original DEHP report				
Summary of DEHP reproductive toxicity studies with oral exposure				
Protocol and study	Reproductive NOAEL (mg/kg bw/day)	Reproductive LOAEL (mg/kg bw/day) and effects	Systemic LOAEL (mg/kg bw/day) and effects	Reproductive effects observed at higher dose levels
Continuous breeding and cross-over mating study in 11-week-old CD-1 mice. 20 pairs of mice were fed diets with DEHP (0, 14, 141, and 425 mg/kg/day) for 7 days prior to mating and during a continuous 98-day mating period [168,186]	Reproductive: 14 Systemic: not known since lower dose groups not examined	141 Reduced fertility ↓ Live pups per litter	425 ↑ Liver weight	Complete infertility in females and reduced fertility in males Seminiferous tubule damage with adverse effects on sperm numbers, motility, and morphology ↓ Male reproductive organ weights
Dose-setting two-generation dietary study in Wistar rats. 10 Pairs/group received 0, 110, 339, or 1060 mg/kg/day for 70 days prior to mating through gestation and lactation [169]	Reproductive: 339 Systemic: none	1060 ↓ Anogenital distance in males ↓ Ovary weight in F_0 ↓ Testes and epididymis weight in F_1 Testicular lesions ↑ Nipple developmental in F_1 males Spermatocyte loss in F_1 ↓ Postnatal survival in F_1 and prenatal survival in F_1 and F_2 pups	110 ↑ Liver weight in F_0 females	No higher doses
90-Day repeat-dose dietary study in Sprague-Dawley rats 10 Rats/sex/group (4–6-week-old) received doses of: M: 0, 0.4, 3.7, 38, 375; F: 0, 0.4, 4.2, 42, 419 [75]	Reproductive: 3.7 (M) Systemic: 3.7–4.2 (M and F)	38 (M) Mild Sertoli cell vacuolation	38 (M)–42 (F) Liver enzyme effects	Atrophy of seminiferous tubules, loss of spermatogenesis, and Sertoli cell vacuolation
1–12-Day repeat-dose gavage study in female Sprague-Dawley rats 6–10 Rats/group (60–70-day-old) received doses of 0 or 2000 mg/kg/day [219]	Reproductive: not known since only one dose administered Systemic: not examined	2000 ↓ Estradiol levels and suppression of ovulation	Not examined	No higher doses

Questions for the Expert Panel

Are human data sufficient for an evaluation of the male or female reproductive toxicity of DEHP?

If so, what are the relevant exposure conditions and endpoints?

How, if at all, are the conclusions about the human reproductive effects of DEHP different from those in the first Expert Panel report?

Are experimental animal data sufficient for an evaluation of the male or female reproductive toxicity of DEHP?

If so, what are the relevant experimental animal models, exposure conditions, and endpoints?

If the experimental animal data are sufficient for an evaluation, are the data assumed relevant, relevant, or not relevant?[a]

How, if at all, are the conclusions about the experimental animal reproductive effects of DEHP different from those in the first Expert Panel report?

[a]The definitions of the term sufficient and the terms assumed relevant, relevant, and not relevant are in the CERHR guidelines at http://cerhr.niehs.nih.gov/news/guidelines.html.

5.0   SUMMARIES, CONCLUSIONS, AND CRITICAL DATA NEEDS

Section 5.0 will be written at the Expert Panel meeting.

5.1   Summary and Conclusions of Reproductive and Developmental Hazards

5.2   Summary of Human Exposure

5.3   Overall Conclusions

5.4   Critical Data Needs

6.0 References

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